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THE DEVELOPMENT OF AN IMMUNOFLUORESCENCE TECHNIQUE AND ITS APPLICATION TO THE RAPID IDENTIFICATION OF COXSACKIE B VIRUSES

BY

RICHARD PING-CHU LAM

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ABSTRACT

The present study was undertaken to develop a reproducible method for the conjugation of fluorescein isothiocyanate (FITC) to immunoglobulins and to assess the sensitivity and specificity of direct immunofluorescence staining for the rapid identification of coxsackie B viruses in tissue culture cells. Immune sera were obtained from guinea pigs immunized with coxsackie B viruses. Type-specific conjugates to coxsackie B viruses were prepared by labelling immunoglobulins with FITC by a dialysis method. The conjugates had weight (μg/mg) fluorescein/protein ratios ranging from 5.0 to 7.1 and estimated molar F/P ratios from 2.1 to 2.9. These conjugates were applied directly to stain HeLa cells infected with coxsackie B viruses. Two types of specific fluorescence were observed in homologous staining: intense perinuclear fluorescent masses and pin-point foci of cytoplasmic fluorescence. Homologous staining titers of the conjugates varied from 1:40 to 1:160, while heterologous staining revealed only low levels of diffuse cytoplasmic fluorescence at conjugate dilution 1:5. At the end-point dilution, the conjugates possessed no heterologous staining reactivity but displayed brilliant (4+) specific staining. The specificity of the fluorescence staining was based on the following observation. Staining occurred only with intracytoplasmic viral antigen homologous to the conjugate. No staining was observed when conjugates were applied to uninfected HeLa cells, and no staining was produced by applying conjugated normal serum to virus-infected cells. Furthermore, specific staining was completely inhibited by pre-treating the preparation with unconjugated immune serum

homologous to the antigen being stained, but not by pre-treatment with heterologous immune sera or normal serum. In the present study, the direct immunofluorescence technique was both sensitive and specific in identifying coxsackie B viruses in tissue culture cells. This technique shows good potential in its application to the detection of viral antigens in human or animal tissues.

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ABBREVIATIONS

AGMK	- African green monkey kidney
DEAE	- diethylamino ethyl
FDA	- fluorescein diacetate
FITC	- fluorescein isothiocyanate
F/P	- fluorescein/protein
HBSS	- Hanks' balanced salt solution
HeLa	- human carcinoma of cervix (cell line)
HEp-2	- human epidermoid carcinoma of larynx (cell line)
IU	- international unit (of antibiotics)
LD_{50}	- 50% lethal dose
MEM	- minimal essential medium
PBS	- phosphate buffered saline
$TCID_{50}$	- 50% tissue culture infectious dose
UFM	- unreacted fluorescent material

INTRODUCTION

The principle of immunofluorescence staining (also known as the fluorescent antibody technique) is based on the fact that, in an antigen-antibody reaction, the antibody becomes bound to its homologous antigen. By labelling the antibody with a fluorescent dye[1] which can be visualized by fluorescence microscopy, the site of the antigen-antibody reaction can be observed. The presence of labelled antibody then designates the location of its homologous antigen.

It has been established that the antibody molecule can be covalently linked with fluorescent dyes without destroying its immunologic specificity. Fluorochromes react with certain chemical groups in the protein molecule, including the amino and carboxyl groups at the ends of each protein chain, amino group in the lysine side-chain, or carboxyl groups in the aspartic and glutamic acid residues (Nairn, 1969). The two most commonly used fluorochromes are fluorescein and lissamine rhodamine B (RB 200). Fluorochromes must have a high fluorescence efficiency to be suitable for preparation of conjugates. Fluorescence efficiency or quantum yield is defined as the ratio of the number of quanta emitted to the number absorbed. The ratio is always less than one because there is more quanta of light absorbed than is emitted in the form of fluorescence. The fluorescence efficiency of fluorescein is 0.70 and that of rhodamine B is 0.25 (Nairn, 1969). The former

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1. A fluorescent dye or fluorochrome can be excited by light of short wavelength (e.g. ultraviolet) to emit visible light of longer wavelength, which can be observed as "fluorescence".

fluorochrome is most frequently used to label serum because of its higher fluorescence efficiency. Furthermore, fluorescein emits a green fluorescence which is sufficiently different to be distinguished from the blue autofluorescence of animal tissue.

Fluorescein has to be converted to a reactive form, without affecting its fluorescent structure, which possesses chemical groups capable of forming stable covalent bonds with protein (Figure 1). The introduction of fluorescein isothiocyanate (FITC) by Riggs et al (1958) has greatly simplified fluorescein conjugation. FITC replaced the previously used fluorescein isocyanate as the fluorochrome of choice by virtue of its stability, lack of toxicity and ease in preparation. The commercial availability of FITC of high purity has been an important stimulus in the field of immunofluorescence.

Conjugation of globulins with FITC is a complex subject. It has been extensively reviewed by Goldman (1968) and Nairn (1969) in their excellent textbooks to which constant references were made throughout this study. In the following review, some details of close relevance to our methodology are presented in order to elucidate the complexities concerning the subject.

Antisera for FITC labelling are prepared according to the same principles as those used in general immunological work. Virus antigens and other kinds of antigens are purified in various ways prior to inoculation into suitable animal hosts in order to reduce contaminant antigens derived from the tissues in which the virus is propagated. A rather large total dosage of antigen is gradually given to the animal host by practically any route other than orally and over a period of several weeks to several months. Crude fractions or highly refined

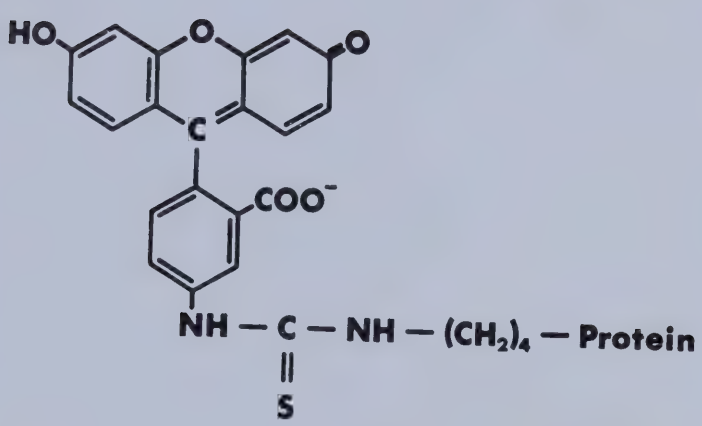

Figure 1

FITC Conjugate with ε-amino Group of a Lysine Residue in a Protein.

globulin fractions are prepared from sera by different methods before FITC labelling. One of the most commonly adopted methods to precipiate globulins from the serum is by ammonium sulfate fractionation. Kaufman and Cherry (1961) first pointed out that contamination of globulin with 0.08 M or greater concentration of ammonium sulfate interfered with conjugation to FITC. On the other hand, Hebert and Pittman (1965) showed that precipitated globulins lost over 99% of their ammonium sulfate within 6 hours of dialysis (in a 27/32-inch bag) against 200 volumes of saline.

Two methods of labelling globulins with FITC are currently used: the direct method and the dialysis method.

With the direct method of labelling, the FITC solution is added directly to the globulin solution. Hebert et al (1972) have described in detail this technique which is used in several laboratories.

The dialysis method was devised by Clark and Shepard (1963). With this method, the globulin solution is dialyzed against the FITC solution so that the dye diffuses through the dialysis membrane into the globulins. Conjugates prepared by this method exhibit considerably less nonspecific staining than conjugates prepared by the direct method, provided that the concentration of FITC did not exceed 20 mg per gram of protein.

All conjugates contain buffer salts which are removed by dialysis. The conjugates also contain various unwanted fluorescent components such as impurities from the original fluorochrome used, hydrolyzed fluorochrome or other products formed during the conjugation reaction. These unwanted fluorescent components are collectively called unreacted fluorescent material (UFM). They must be removed from the conjugates

since they constitute an important source of nonspecific staining. UFM is best removed by gel filtration through a Sephadex G-25 or G-50 column (Nairn, 1969).

McKinney et al (1964a) have extensively studied the factors that control globulin labelling with FITC. They reported that conjugation of rabbit immunoglobulins was accurately controlled by the amount of FITC used. The conjugation reaction was essentially completed in 30 minutes if the reaction temperature was at 25°C, the protein concentration at 2.5% (25 mg/ml) and if a 0.05 M phosphate buffer (pH 9.5) was used. They indicated that conjugation at 25°C allowed much more efficient utilization of FITC and consequently more reproducible results. However, these workers were testing conjugates on bacterial smears only. They did not therefore discuss nonspecific staining frequently encountered with tissue culture cells or sections of animal tissue.

Immunofluorescence staining in these host systems was investigated by Spendlove (1966). He conjugated anti-reovirus immunoglobulins from several animal species with varying concentrations of FITC to determine whether the optimal concentration of FITC to be used in conjugation was different for each immune serum. He had assumed that globulins of high antibody titer could be labelled more heavily since they could be diluted further to eliminate nonspecific staining. The results obtained, however, showed that conjugation conducted at the proportion of 20 mg of FITC per gram of protein was optimal for all sera. Use of FITC at higher concentrations (even at 21 mg per gram of protein) would lead to excessive nonspecific staining which kept increasing with the concentration of the dye. To obtain more uniform labelling and eliminate the occurrence of "pockets" of high FITC concentration, Spendlove devised a modification

of the direct method of labelling. He adjusted the pH to 9.5 after FITC was mixed with the serum proteins.

Stainingreactions with FITC conjugates are relatively simple to perform. The same basic procedures are applied whether information is sought on the antigen or the antibody. Since the purpose of this study is to identify viral antigens, the methods employed to demonstrate unknown antigens are hereby described. Unknown antigen is detected by means of known antibodies, using either the direct method or the indirect method of staining. There is also the complement method of staining which is less frequently used.

The direct method of staining was devised by Coons et al (1942) who introduced the immunofluorescence technique and was modified later by Coons and Kaplan (1950). Specific immunoglobulins are labelled with FITC and the conjugate is directly used to stain microscopic preparations containing the homologous antigen.

The indirect method of staining was introduced by Weller and Coons (1954). It is a two-stage procedure in which the antigen preparation is first treated with homologous antiserum and subsequently the antigen-bound globulin is stained by means of labelled animal-species-specific antiglobulin.

The complement staining method was introduced by Goldwasser and Shepard (1958). This method is based on the fact that most antigen-antibody reactions bind complement. The antigen is first treated with a mixture of inactivated unlabelled antiserum and guinea pig complement. Thereafter, a FITC-conjugated anti-guinea pig complement is applied. The disadvantage of this method lies in the number of controls required to establish the specificity of any fluorescence produced, since any of

the three reagents involved may be a source of nonspecific staining.

When the immunofluorescent technique was introduced by Coons et al in 1942, it seemed promising to revolutionize the diagnostic methods of viral diseases. It permits the rapid identification of specific viral antigens and requires relatively simple equipment within the capacity of most virus diagnostic laboratories. However, despite successful applications to the diagnosis of influenza by Liu (1956) and of rabies by Goldwasser and Kissling (1958), the technique has not lived up to expectation in diagnostic virology. The recent publication of a monograph by Gardner and McQuillin (1974) should encourage its general use.

Our interest in the diagnosis of coxsackie B viruses (types 1 to 6) by immunofluorescence originated from their role as a possible causative agent of chronic cardiac diseases (Lerner and Wilson, 1973). Like the morphologically similar coxsackie A viruses, polioviruses and echoviruses, coxsackie B viruses are enteroviruses in the picornavirus group. Complete virions are 18-25 nm in diameter, being composed of a single-stranded RNA molecule enclosed in a naked protein capsid with 32 capsomers. Cubic symmetry of the icosahedral type has been suggested as the structural form (Melnick and Wenner, 1969). Virus replication appears to take place in the cytoplasm of host cells (Levintow, 1974).

Immunofluorescence staining has not found wide application to the rapid identification of enteroviruses. Buckley (1956, 1957) was one of the first investigators to show, by immunofluorescence, the sequence of events in tissue culture after infection with polioviruses. She described in detail the morphology of the newly formed viral antigen at different stages after infection.

Shaw et al (1961) reported the use of a direct immunofluorescent technique to identify certain coxsackieviruses and echoviruses. Rabbit antisera to these viruses were pooled according to the scheme of Lim and Benyesh-Melnick (1960). The serum pools were conjugated with FITC, then absorbed with rabbit brain, liver powders and monkey kidney cells to reduce nonspecific staining. With the help of rhodamine B as counter-stain, a rapid although provisional identification of the enteroviruses in tissue culture was achieved.

Hatch et al (1961) and Hatch (1963) employed the direct immuno-fluorescent technique to identify enteroviruses, including coxsackie B viruses in monkey kidney cells inoculated with stool specimens. FITC was conjugated to type-specific antisera. The conjugates were absorbed with monkey liver powders and monkey kidney cells to reduce nonspecific staining. Infected cultures were stained at an early stage of cytopathic change. All isolates identified by the immunofluorescent method were confirmed by neutralization tests and the technique was regarded as highly specific.

Zalan et al (1965) applied the indirect immunofluorescent tech-nique to identify group A coxsackieviruses propagated in primary human amnion cell cultures. Some cross-reactions were noted between some of the serotypes but they could be easily differentiated from type-specific reactions by the intensity of fluorescence. Nonspecific staining was reduced by absorption of the antisera with mouse liver powder and human amnion cells.

Sommerville and Macfarlane (1964) utilized the indirect immuno-fluorescent technique to detect group B coxsackieviruses in human leuko-cytes and in cells from cerebrospinal fluid (Sommerville, 1966).

Specific fluorescence was reported but the results await confirmation.

Contrary to the type-specific immunofluorescence reported by previous workers, Chaudhary and Westwood (1970) observed strong cross-reactions within and between the poliovirus, coxsackievirus and echovirus groups. Using the indirect immunofluorescent technique, viruses were grown in green monkey kidney cells and demonstrated by rabbit antisera together with conjugated guinea pig anti-rabbit globulin. They concluded that the cross-reactions represented a group-specific response as the result of shared specificity within the enterovirus group.

The most noteworthy report to date on the immunofluorescence staining of group B coxsackieviruses was by French and co-workers (1972). The sensitivity and specificity of the indirect immunofluorescent technique were assessed. Antisera produced in monkeys and hamsters and immune ascitic fluids from mice were tested for homologous and heterologous staining of BS-C-1 cell cultures infected with group B coxsackieviruses. Acetone-fixed microscopic smears were prepared from trypsinized culture cells. Each smear was treated with two-fold dilutions of immune sera, then stained with a "working dilution" of FITC-conjugated anti-species globulin. Immune reagents from these species showed some heterologous and nonspecific staining at low dilutions. However, no consistent patterns of heterologous staining were demonstrated. It was observed that the immune reagents could be diluted to a point where they gave no heterologous reactivity, but still showed characteristic homologous staining. Using an appropriately diluted hamster antiserum, this group of workers reported the correct identification of 79 (93%) from 85 field strains of group B coxsackieviruses after one passage in BS-C-1 cells, while the remaining strains were identified after two passages. Nevertheless, they

failed to detect homologous staining in excess of nonspecific or heterologous staining in two human heart and brain tissues from which coxsackievirus B4 had been isolated. This report presented encouraging results in the use of indirect immunofluorescence staining for identifying group B coxsackieviruses in tissue culture, but the technique failed in its application to human tissues. Moreover, the methodology was not sufficiently standardized. No fluorescein/protein ratio of the conjugates was mentioned, nor was the "working dilution" of the conjugates quantitatively explained. More significantly, the specificity of the homologous staining had not been confirmed by antibody blocking tests.

The etiological role of coxsackie B viruses in cardiac diseases was extensively studied by Burch et al (1967, 1968). They demonstrated, by direct and indirect methods of immunofluorescence staining, the localization of coxsackie B virus antigens in human heart tissues taken at autopsies. In most instances, however, staining of heart tissues by immunofluorescence showed positive results with antisera to more than one type of coxsackie B viruses. Their supporting evidence was not entirely satisfactory for the lack of proper controls.

The ultimate goal of this investigation is to assess the role of coxsackie B viruses in the etiology of human myocardiopathy. In anticipation of the complexities of tracing viral antigens in animal tissues in vivo, the parameters of immunofluorescence staining must first be explored in vitro using an experimental host system of tissue culture cells. The present study was therefore undertaken to develop a reproducible method for the conjugation of FITC to immunoglobulins and to assess the sensitivity and specificity of direct immunofluorescence staining for the rapid identification of group B coxsackieviruses in

tissue culture cells. Such a study is essential prior to applying the immunofluorescent technique for the detection of coxsackie B viruses in animal tissues.

MATERIALS AND METHODS

1. Virus Strains

The following certified virus strains of group B coxsackieviruses were employed throughout this study.

Coxsackievirus B1 (Connecticut 5)

Coxsackievirus B2 (Ohio 1)

Coxsackievirus B3 (Nancy)

Coxsackievirus B4 (JVB)

Coxsackievirus B5 (Faulkner)

Coxsackievirus B6 (Schmitt)

The coxsackievirus B3 strain was obtained from Dr. M. H. Hatch, Enteric Virology Unit, National Communicable Disease Center, Atlanta, Georgia. All the other strains were obtained from the Research Reference Reagent Laboratory, National Institute of Health, Bethesda, Maryland.

2. Tissue Culture Cells

Two lines of human cells, both obtained from Flow Laboratories, were employed. The HEp-2 cells (human epidermoid carcinoma of larynx) were used primarily for the propagation of viruses, whereas the HeLa cells (human carcinoma of cervix) were used for virus propagation, virus titration and the preparation of coverslip cultures.

In addition, primary African green monkey kidney cells (AGMK) obtained from Connaught Laboratories were used for virus propagation, titration as well as neutralization tests.

3. Tissue Culture Media

The growth medium for HEp-2 and HeLa cells consisted of Eagle's minimal essential medium (MEM, with Earle's salts) supplied by Flow Laboratories. It was supplemented with 10% fetal bovine serum, 100 IU/ml of penicillin-G, 100 µg/ml of streptomycin sulfate, 15 IU/ml of mycostatin, and was adjusted to pH 7.4 with 7.5% sodium bicarbonate.

Eagle's MEM supplemented with 2% fetal bovine serum, 0.5% lactalbumin hydrolysate (Grand Island Biological Co., Inc.) and antibiotics was used for the growth of primary AGMK cells.

For each type of cell culture, the maintenance medium was equivalent to the growth medium without fetal bovine serum.

4. Propagation of Viruses

The viruses used for the preparation of immune sera (designated as vaccine viruses) were propagated four times in primary AGMK cells and subsequently three times in HEp-2 cells. This was followed by a final passage in HeLa cells because at that particular time HEp-2 cells were not readily available. These vaccine viruses were labelled as $AGMK_4/HEp\text{-}2_3/HeLa_1$.

The viruses used for the infection of coverslip cultures (designated as seed viruses) were propagated four times in primary AGMK cells followed by four passages in HEp-2 cells, and were labelled as $AGMK_4/HEp\text{-}2_4$.

Eight Roux bottles containing monolayers of culture cells were routinely used for each virus passage. Prior to inoculation, the tissue culture growth medium was discarded. Ten ml of a virus suspension, containing approximately $10^5 TCID_{50}/0.1$ ml (tissue culture infectious dose), were inoculated into each bottle culture. The virus was allowed to adsorb to the culture cells for 60 minutes at 37^oC. After the adsorption

period, 100 ml of tissue culture maintenance medium were added. The infected cells were incubated at 37°C and harvested when complete cell degeneration (4+ cytopathic effect) occurred, usually after 24 to 48 hours of incubation.

The infected cultures were frozen at -70°C and thawed at room temperature for three consecutive times. Thereafter, the content of each Roux bottle was pooled. The pooled culture fluid was centrifuged at 8,000 rpm for 60 minutes at 4°C in a Sorvall RC-2 centrifuge with a SS-34 rotor to remove cellular debris. The virus-containing supernatant was treated with fluorocarbon (Genesolv-D, trifluorotrichloroethane, Allied Chemical) according to the method of Hamparian et al (1958). One volume of fluorocarbon was added to two volumes of virus suspension and the mixture was homogenized while the treatment vessel was submerged in an ice bath. The mixture was kept at 4°C overnight to allow separation of the organic phase from the aqueous phase. The virus-containing aqueous phase was subsequently removed and centrifuged as described above. The supernatant was decanted, pooled and assayed for viral infectivity. The virus preparation was preserved at -70°C.

5. Titration of Virus Infectivity

The amount of infectious virus present in the fluorocarbon-treated virus suspensions was titrated in tissue culture according to standard methods (Schmidt, 1969). After the final passage, the vaccine virus preparations ($AGMK_4/HEp\text{-}2_3/HeLa_1$) were titrated in HeLa cells because other cell types were not available at that time. The seed virus preparations ($AGMK_4/HEp\text{-}2_4$) were titrated in primary AGMK cells and HeLa cells.

In each titration, ten-fold dilutions of virus were prepared with tissue culture maintenance medium. Each virus dilution, in 0.1 ml amounts, was inoculated into four tubes of culture cells. The inoculated cultures were incubated at 37°C and examined daily for cytopathic effect (degeneration) over a 7-day period. The 50% end-point was determined by calculating the highest virus dilution which produced cytopathic effect in 50% of the cell cultures inoculated (Reed and Muench, 1938). The virus titer was expressed in $TCID_{50}$ per 0.1 ml (50% tissue culture infectious dose).

Attempts were made to titrate the viruses in suckling mice (one day old). Virus dilutions were prepared as described above and 0.02 ml of each dilution was inoculated intracerebrally into eight suckling mice. Inoculation via the intraperitoneal route (0.04 ml per mouse) was also attempted. The inoculated mice were examined for mortality over a period of 14 days.

6. Preparation of Immune Sera

Female guinea pigs (age 3 to 4 months, weighing 350 to 450 g) were used as host animals. Prior to immunization, the guinea pigs were bled for normal serum (designated as pre-immunization serum). Each animal received a series of three injections of undiluted virus suspension (approximately $10^6 TCID_{50}$/0.1 ml) at weekly intervals via the following routes: 1.0 ml intraperitoneally and 0.5 ml subcutaneously (into the interscapular brown fat). In the fourth week, each animal received 1.5 ml of a virus-adjuvant mixture[2] intraperitoneally and 0.5 ml

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2. The adjuvant was prepared by mixing 9 volumes of paraffin oil with 1 volume of Arlacel A (mannide monooleate, Atlas Chemical Industries, Inc.). Equal volumes of virus suspension and the adjuvant were then combined and shaken until homogenous to obtain the virus-adjuvant mixture.

subcutaneously. The animals were test-bled in the sixth week. During the seventh and eighth week, each animal was given booster injections of 2.0 ml of the virus-adjuvant mixture intraperitoneally and 1.0 ml subcutaneously.

The animals were bled out by cardiac puncture approximately four weeks after the last injection. The immune serum was separated from the clots and centrifuged at 8,000 rpm for 60 minutes at 4°C in a Sorvall RC-2 centrifuge with a SS-34 rotor. The supernatant serum was pooled and preserved at -70°C until use.

In this manner, immune sera to each of the coxsackie B viruses were obtained. A batch of normal serum was prepared by bleeding out non-immunized guinea pigs. This normal (non-immune) serum was used for the preparation of a control FITC-conjugate. The pre-immunization sera, because of their small quantity, were reserved for performing antibody blocking tests.

7. Determination of Neutralizing Antibody Titers

The level of neutralizing antibodies in each anti-coxsackie B virus immune serum obtained from guinea pigs was determined by neutralization test, using primary AGMK tube cultures as host system. Neutralization titers were determined by the constant virus-varying serum method, in which dilutions of serum were tested against a constant dose of virus. The method of Melnick and Wenner (1969) was used. The immune sera were absorbed with HeLa cells which were used for the propagation of vaccine viruses. Immune sera before and after HeLa cell absorption were tested in parallel to detect the effect of anti-HeLa cell antibodies on the neutralization titer.

Before each test, the serum was inactivated at 56°C for 30 minutes to remove nonspecific inhibitors of virus infectivity. The challenge virus consisted of a constant dose of 100 $TCID_{50}$/0.1 ml of tissue culture-passaged seed virus ($AGMK_4$/$HEp\text{-}2_4$) from the respective coxsackie B virus strain. Ten-fold virus dilutions and two-fold serum dilutions were then prepared with tissue culture maintenance medium. Virus in 1 ml amounts, calculated to contain 100 $TCID_{50}$ per 0.1 ml, was added to an equal volume of each serum dilution. The mixtures were incubated at 37°C for 60 minutes, then inoculated in 0.2 ml amounts into each of four culture tubes per serum dilution. A back titration of virus titer was conducted using 100, 10 and 1 $TCID_{50}$/0.1 ml of virus. The cultures were incubated at 37°C and examined daily for seven days to detect cytopathic effect. The neutralization titer was determined as the highest serum dilution which "protected" the cultures against the challenge virus.

Attempts were also made to determine the neutralization titers of the HeLa cell-absorbed immune sera in suckling mice (one day old) using a constant virus dose of 100 LD_{50} (lethal dose) per 0.02 ml with increasing dilutions of immune serum (Melnick and Wenner, 1969). The same principle was applied and virus-serum mixtures were set up in the same way as just described. The mixtures were inoculated in 0.04 ml amounts intraperitoneally into eight suckling mice per serum dilution. The mice were checked daily for mortality for 14 days.

8. Precipitation of Immunoglobulins with Saturated Ammonium Sulfate

Serum fractionations were performed with saturated ammonium sulfate (760 g per liter) adjusted to pH 7.0 with 1 N sodium hydroxide

(Sinha and Reddy, 1964). Ten ml of immune serum were diluted with 10 ml of deionized water. Subsequently 20 ml of saturated ammonium sulfate solution were added dropwise with stirring at 4°C. The mixture was kept at 4°C overnight and then centrifuged at 8,000 rpm for 60 minutes at 4°C in a Sorvall RC-2 centrifuge with a SS-34 rotor. The supernatant was discarded and the packed globulins were dissolved in deionized water to a final volume of 10 ml. A second precipitation was performed similarly by adding 10 ml of saturated ammonium sulfate to the dissolved globulins. The mixture was centrifuged immediately as described above and the final precipitate was dissolved in 0.85% sodium chloride to a final volume of about 5 ml. The reconstituted globulins were dialyzed, in a 3/4-inch diameter dialysis tubing, against 4 liters of 0.85% sodium chloride (adjusted to pH 7.0) at 4°C with four to five changes of saline over a two-day period to remove traces of ammonium sulfate. The dialysis was considered complete when no cloudiness was observed in mixing equal volumes of the dialyzing saline and a saturated barium chloride solution.

9. Determination of Protein Content by the Biuret Reaction

The protein in the dialyzed globulin sample was determined by the biuret reaction. The biuret reagent was prepared according to Gornall et al (1949). A protein standard curve was prepared for each batch (500 ml) of biuret reagent, using rabbit gamma globulin, fraction II (Miles Laboratories), as the standard. The standard protein was dissolved in PBS, pH 7.2, at 5 different concentrations ranging from 0.3 mg/ml to 5 mg/ml. Four milliliters of biuret reagent were added to 1 ml of each protein solution and to 1 ml of PBS, pH 7.2, as blank. The samples were incubated at room temperature for 30 minutes to develop

color. The absorbance of each sample was read in 1.0 cm cells against the blank at 540 and 560 nm on a Beckman Model 25 spectrophotometer, the wavelength calibration and absorbance of which had been checked prior to each determination. A linear standard curve was obtained by plotting absorabance at 560 nm against protein concentration (mg/ml). In measuring the protein content of a globulin solution, the sample was diluted 1:10 and 1:20 with PBS, pH 7.2. Then 0.5 ml of each dilution was mixed with 2.0 ml of biuret reagent and the samples were incubated at room temperature for 30 minutes. A blank was set up by adding 2.0 ml of biuret reagent to 0.5 ml of PBS, pH 7.2. The absorbance readings of the samples were observed as described above against the blank. The protein content of each globulin dilution was determined in duplicate and the average value was converted to milligrams of protein per ml of the undiluted globulin solution.

10. Conjugation of Immunoglobulins with Fluorescein Isothiocyanate

The immunoglobulins were conjugated with crystalline fluorescein isothiocyanate (FITC, Baltimore Biological Laboratory, Baltimore, Maryland) by a modification of the dialysis technique of Clark and Shepard (1963).

Conjugation was conducted in the proportion of 18 mg of FITC per gram of protein. The required amount of FITC was dissolved in 0.1 M Na_2HPO_4 and the pH was adjusted to 9.5 with 0.1 M Na_3PO_4. Meanwhile, a calculated amount of immunoglobulin solution was diluted to a concentration of 2% protein (20 mg/ml) with 0.2 M Na_2HPO_4, then adjusted to pH 9.5 with 0.1 M Na_3PO_4 (Hebert et al, 1972).

For labelling, the FITC solution was placed in a dialysis tubing

(1/4-inch inflated diameter) and submerged into the immunoglobulin solution. Conjugation was allowed to proceed at room temperature for 4 hours with constant stirring by a magnetic stirrer. Thereafter, the conjugation procedure was discontinued by discarding the dialysis tubing containing the dye solution. The labelled immunoglobulins were then dialyzed, in a 3/4-inch diameter dialysis tubing, against 4 liters of 0.85% sodium chloride (adjusted to approximately pH 8.5 with 1 N sodium hydroxide) at 4°C with 6 to 7 changes of saline over a 3-day period. After dialysis, the conjugate was kept at -70°C until further treatment.

11. Absorption with HeLa Cells

The dialyzed conjugate was absorbed with HeLa cells to remove unwanted antibodies. HeLa cell monolayers grown in Roux bottles were dispersed with 0.25% trypsin (Difco Laboratories), suspended in tissue culture maintenance medium and packed at 1,200 rpm in a Model K International Centrifuge (total volume of packed cells was approximately 0.5 ml). The cells were washed with PBS, pH 7.2, and packed in a graduated centrifuge tube successively for three times. After the last packing, the cells were re-suspended in 3 ml of conjugate. Absorption of the conjugate with the washed cells was allowed to proceed for 30 minutes at room temperature, with frequent agitations, and then at 4°C overnight. The conjugate-cell mixture was centrifuged at 8,000 rpm for 60 minutes at 4°C in a Sorvall RC-2 centrifuge with a SS-34 rotor. The supernatant conjugate was preserved.

12. Sephadex G-50 Gel Filtration

The conjugate was further freed of uncombined FITC by passage through a Sephadex G-50 column (Zwaan and van Dam, 1961). A 12 mm

x 350 mm column was packed with Sephadex G-50 Fine (Pharmacia, Sweden) gelated in PBS, pH 7.2, at room temperature. A 3 ml sample of the conjugate was applied to the column and eluted with PBS, pH 7.2. Two yellow-colored bands were observed (with the naked eye) in the column, separated by a distance of approximately 10 cm. The first band contained the protein-dye complex and no free dye; the second contained free dye only. The first colored fraction to emerge from the column was collected.

The FITC-conjugate fraction collected from the Sephadex G-50 column was passed through a 200 nm pore-diameter membrane filter to achieve bacterial sterility. Several samples of the same conjugate purified by gel filtration were pooled and assayed for fluorescein/protein ratio.

The above procedures (ammonium sulfate precipitation, conjugation, HeLa cell absorption and Sephadex gel filtration) were employed to prepare conjugates from type-specific immune sera to each of the coxsackie B viruses. For each virus type, the same batch of immune serum was used throughout the experiments. A normal (control) conjugate was also prepared from normal (non-immune) guinea pig serum.

13. Determination of Fluorescein/Protein Ratio

After Sephadex gel filtration, the conjugate was characterized by determining its fluorescein/protein ratio (F/P).

(a) Protein Determination

The total protein concentration (mg/ml) in the conjugate was measured by the biuret method, as previously described. Absorbance was read in 1.0 cm cells at 560 nm on a Beckman Model 25

spectrophotometer.

(b) FITC Determination

Fluorescein isothiocyanate (FITC) was determined as protein-bound FITC by absorbance in 1.0 cm cells in 0.1 N sodium hydroxide at 490 nm (wavelength of maximum absorption) on a Beckman Model 25 spectrophotometer. The reference standard for FITC determination was fluorescein diacetate, FDA[3], dissolved in 0.1 N sodium hydroxide (McKinney et al, 1964b). A reference standard curve was established by measuring the absorbance in 1.0 cm cells at 490 nm of FDA dissolved in 0.1 N sodium hydroxide at various concentrations between 1 and 3 µg of FDA/ml. To measure the amount of protein-bound FITC, the conjugate was appropriately diluted with 0.1 N sodium hydroxide and the absorbance was read at 490 nm against the diluent. FDA concentration values obtained from the standard curve were converted to protein-bound FITC concentration by multiplying with the factor 1.07, e.g. FDA µg/ml x 1.07 = protein-bound FITC µg/ml. When multiplied by the dilution required to read the absorbance, the calculated value was the concentration of protein-bound FITC in the conjugate in µg/ml.

The relationship was based on the following: For a given spectrophotometer, the extinction coefficient (E_1 µg/ml) for FDA (0.208) divided by that for protein-bound FITC (0.195) is the constant 1.07. The use of this factor and a standard curve prepared with FDA

3. Supplied by Eastman Kodak Co., Rochester, New York.

eliminates the effect of variation in spectrophotometers and relates all measurements of protein-bound FITC to the primary reference standard, FDA (Hebert et al, 1972).

(c) Calculation of F/P Ratio

A weight/weight ratio of fluorescein to protein was obtained by dividing the protein-bound FITC concentration (µg/ml) by the protein concentration (mg/ml), and was expressed as µg FITC per mg protein (µg/mg). After the weight ratio was calculated, it was converted to an estimated molar ratio by multiplying with the factor 0.411. The latter was derived from the expression

$$\frac{160{,}000 \times 10^{-3}}{389} = 0.411$$

where 160,000 was taken as the average molecular weight of gamma globulin, 389 as the molecular weight of FITC and 10^{-3} converts mg's to µg's of FITC (Hebert et al, 1972).

(d) Storage of Conjugates

After F/P ratio determination, the conjugates were dispensed in 0.5 ml quantities and stored at -70°C until use.

14. Preparation of Virus-infected Coverslip Cultures

HeLa cells were grown on glass coverslips (8 mm x 30 mm, Corning Glass Works) in Leighton tubes until a full monolayer was obtained. Each tube was inoculated with approximately 2×10^5 $TCID_{50}$ of virus contained in 0.2 ml of inoculum. The virus was allowed to adsorb to the HeLa cells at 37°C for 60 minutes. Control HeLa cells were inoculated under similar conditions with 0.2 ml of maintenance medium. After adsorption, each coverslip was rinsed once with 2 ml of Hanks' balanced salt solution

(HBSS), pH 7.4, to remove unadsorbed virions. One ml of maintenance medium was then added to each tube and the cells were incubated at 37°C. After approximately 12-16 hours of incubation, a 2+ to 3+ cytopathic effect was observed (50 to 75% of the total number of cells showed signs of degeneration). During harvest, each coverslip was rinsed with 2 ml of HBSS, pH 7.4, then removed from the tube and air-dried at room temperature. The coverslips were subsequently fixed in acetone at room temperature for 10 minutes. The fixed coverslips were air-dried and preserved at -70°C. For each batch of coverslips prepared, an equal number of control coverslip cultures were prepared under similar conditions.

15. Direct Immunofluorescence Staining

Immediately before being tested for specific and nonspecific staining, each conjugate was diluted to a uniform protein concentration of 10 mg/ml with PBS, pH 7.2. Two-fold dilutions (starting at 1:5) of the conjugate were then prepared in a 10% suspension of normal mouse brain in PBS, pH 7.2 (Lennette et al, 1975).

The direct method of immunofluorescence staining was employed throughout the experiments. Coverslips were overlaid with appropriate dilutions of the conjugate in a moist chamber for 30 minutes at room temperature, then rinsed with PBS, pH 7.2. The coverslips were subsequently counterstained for 5 minutes with an aqueous solution of 0.05% Evans blue (Fisher Scientific Co.). The counterstain was thoroughly rinsed away with PBS, pH 7.2, and the coverslips were dipped in distilled water to remove salts. Each coverslip was drained of excess water and mounted, cell-side down, in a drop of buffered glycerine on a 1 mm thick

glass slide. The buffered glycerine, pH 9.5, was prepared by combining 9 volumes of glycerine with 1 volume of 0.1 M Na_3PO_4.

The preparations were examined immediately with a Zeiss binocular dark-field fluorescent microscope. A high pressure mercury lamp, the HBO 200 manufactured by the Osram Company in Munich, was used to provide ultraviolet illumination of high intensity. A Zeiss KP 500 interference filter was used as the primary filter to achieve separation between the maximum absorption and emission wavelengths (495 and 520 nm respectively) for FITC (Lewis et al, 1973). A secondary filter, the Zeiss 50, was used between the objective and the eyepiece to permit only transmissions of wavelengths above 500 nm, allowing the green emission of FITC-conjugates and the red emission of Evans blue counterstain. A non-fluorescent immersion oil (Zeiss) was applied between the condenser and the slide; 12.5x oculars and 10x, 40x (dry) objectives were used for reading.

For each infected coverslip examined by direct immunofluorescence staining, an uninfected coverslip from the same batch was stained in parallel as a negative control.

The degree of fluorescence staining was expressed from 1+ to 4+. A reading of 4+ indicated brilliant yellow-green fluorescence and 3+ indicated bright green but not brilliant fluorescence. Dull green fluorescence was assigned a 2+ or 1+ reading. On this basis, the highest dilution of a conjugate which produced a 3+ or 4+ staining reaction was selected as the end-point titer of the conjugate.

16. End-point Titration of Immunofluorescence Staining and Assessment of Cross-reactivity

The homologous staining titer of each anti-coxsackie B virus conjugate was determined by the direct method of immunofluorescence staining, using coverslip cultures infected with the same type of coxsackie B virus employed to prepare the conjugate. For example, anti-coxsackievirus B1 conjugate was used to stain coverslip cultures infected with coxsackievirus B1.

The heterologous staining reactivities of each conjugate were assessed similarly, using coverslip cultures infected with the other five types of coxsackie B viruses heterologous to the conjugate. For instance, anti-coxsackievirus B1 conjugate was used to stain coverslips infected with coxsackieviruses B2-B6. As a positive control in each heterologous staining, a coverslip from the same batch being tested was stained with the homologous conjugate (homologous to the virus type with which the cells had been infected) in order to demonstrate the presence of the specific viral antigen. For example, when anti-coxsackievirus B1 conjugate was tested for cross-reactivity with coxsackievirus B4 antigen, a positive control was included in which coxsackievirus B4 antigen was stained with the end-point dilution of anti-coxsackievirus B4 conjugate. This positive control ensured that coxsackievirus B4 antigen was present in the infected cells and could be demonstrated with anti-coxsackievirus B4 conjugate at its end-point dilution.

17. Blocking Tests for Specific Staining

To establish that any observed staining was immunologically specific, antibody blocking tests were performed by the sequence (two-step) method of Coons and Kaplan (1950). An undiluted, unconjugated immune

serum homologous to the antigen being stained was applied to virus-infected HeLa cells. The preparations were incubated at room temperature in a moist chamber for 60 minutes and rinsed thoroughly with PBS, pH 7.2. The rinsed preparations were drained of excess rinsing buffer and stained for 30 minutes with dilutions 1:5 to 1:80 of the conjugate homologous to the antigen being stained. The usual procedure of rinsing, counterstaining and mounting was followed. Inhibition of specific staining was examined under the fluorescence microscope.

In this manner, the staining specificity of each conjugate was assessed by blocking tests using undiluted immune sera homologous and heterologous to the antigen being stained. For each blocking test, positive staining controls were included. Virus-infected HeLa cells were treated with normal (pre-immunization) serum and stained as described above.

18. Photomicrography

Photomicrographs of immunofluorescence staining preparations were taken with a Zeiss Type CS camera. Two kinds of 35-mm color slide films were used according to the recommendation of Elliot et al (1974): Kodak High Speed Ektachrome Daylight (ASA 160) and Anscochrome 500 (ASA 500). An exposure time of 30-60 seconds was used. Color films were processed and made into prints by commercial photo-finishers.

19. Formula for Preparing Phosphate Buffered Saline

Phosphate buffered saline, pH 7.2, was prepared according to the protocol of Dr. J. L. Riggs, Viral and Rickettsial Disease Laboratory, Department of Public Health, State of California.

Phosphate Buffered Saline (PBS), pH 7.2, 0.005 M

(a) Concentrated (10x) Stock Solution

NaCl	85 gm
Na_2HPO_4 (anhydrous)	5.65 gm
KH_2PO_4	1.35 gm

Distilled water to a final volume of 1000 ml

(b) Working Solution - PBS, pH 7.2, 0.005 M

Concentrated Stock Solution	100 ml
Distilled water to a final volume of	1000 ml

RESULTS

1. Virus Infectivity Titers

Vaccine virus preparations ($AGMK_4/HEp\text{-}2_3/HeLa_1$) showed $TCID_{50}$ titers of approximately 10^6 per 0.1 ml when titrated in HeLa cell cultures. Titers of seed virus preparations ($AGMK_4/HEp\text{-}2_4$) were approximately the same whether determined in HeLa cells or in primary AGMK cells. The results of seed virus titration in primary AGMK cells are summarized in Table 1. The calculation of a constant virus dose of 100 $TCID_{50}$/0.1 ml was based on the titers reported in Table 1.

Attempts to titrate the vaccine virus and seed virus preparations in suckling mice (one day old) failed because the animals were not susceptible to infection. No animal infectivity titers were obtained.

2. Neutralizing Antibody Titers

The neutralizing antibody titers of the immune sera after HeLa cell absorption are shown in Table 2. Neutralizing antibody titers determined before HeLa cell absorption of the immune sera were similar to that determined after HeLa cell absorption, indicating that the presence of unwanted antibodies had no observable effect on the results of neutralization tests. However, neutralizing antibody titers could not be determined in mice because the mice were not susceptible to infection by the challenge virus. Normal (non-immune) guinea pig serum and pre-immunization serum were shown to have no neutralizing antibody activity.

TABLE 1

TITERS OF SEED VIRUSES ($AGMK_4$/$HEp\text{-}2_4$)

FOR COVERSLIP CULTURE INOCULATION

Coxsackieviruses	$TCID_{50}$ per 0.1 ml (in primary AGMK cells)
B1	$10^{5.5}$
B2	$10^{6.0}$
B3	$10^{6.0}$
B4	$10^{6.0}$
B5	$10^{6.3}$
B6	$10^{6.3}$

$TCID_{50}$ per 0.1 ml: 50% tissue culture infectious dose per 0.1 ml

TABLE 2

NEUTRALIZING ANTIBODY TITERS OF ANTI-COXSACKIE B VIRUS GUINEA PIG IMMUNE SERA (AFTER HeLa CELL ABSORPTION)

Immune Sera to Coxsackievirus	Neutralizing Antibody Titers[a]
B1	10,240
B2	5,120
B3	5,120
B4	20,480
B5	20,480
B6	20,480

a: Reciprocal of the highest dilution of immune serum which neutralized 100 $TCID_{50}$/0.1 ml of the homologous coxsackie B virus.

3. Fluorescein/Protein Ratios of Conjugates

For each type of coxsackie B virus, immune serum obtained from the same batch of guinea pigs was used throughout the entire study for preparing FITC-conjugates. Since immunoglobulins were labelled in small quantities, several conjugates had been prepared from each type-specific immune serum. Conjugates prepared from the same immune serum exhibited very little differences in fluorescein/protein ratio, whereas those prepared from different antisera varied slightly in F/P. Table 3 summarizes the F/P ratios of conjugates to each of the coxsackie B viruses and also to the normal (non-immune) serum. The conjugates reported were those used in the final testing. The weight/weight ratios varied from 5.0 to 7.1, while the estimated molar ratios ranged from 2.1 to 2.9. Based on the latter ratios, approximately two to three molecules of FITC were conjugated to each globulin molecule. Under standardized experimental conditions, the degree of conjugation was shown to be relatively constant. The modified dialysis technique described in this study therefore represented a reproducible method for the conjugation of FITC to immunoglobulins.

4. Direct Immunofluorescence Staining of Virus-infected HeLa Cells

Virus-infected HeLa cells were stained with conjugates to each of the coxsackie B viruses by the direct method of immunofluorescence staining. The intensity of fluorescence was expressed on a four-point scale. A reading of 4+ denoted brilliant yellow-green fluorescence, and 3+ denoted bright green but not brilliant fluorescence. A reading of 2+ or 1+ indicated only dull green fluorescence. Each coverslip preparation was examined under at least 12 microscopic fields. The reading assessed therefore represented the overall intensity of the fluorescence observed.

TABLE 3

FLUORESCEIN/PROTEIN RATIOS OF CONJUGATES

Conjugates to	Fluorescein/Protein Ratio	
	µg/mg	Molar
Coxsackievirus B1	6.5	2.7
Coxsackievirus B2	6.3	2.6
Coxsackievirus B3	5.2	2.1
Coxsackievirus B4	6.5	2.7
Coxsackievirus B5	7.1	2.9
Coxsackievirus B6	6.7	2.8
Normal serum	5.0	2.1

Figures 2 to 5 illustrate the appearance of coxsackievirus B4-infected and uninfected HeLa cells stained with anti-coxsackievirus B4 conjugate at dilution 1:40. The brilliance of the yellow-green fluorescence demonstrated in infected cells was slightly diminished when the original 35-mm color slides were made into prints. Bright 4+ staining reactions were shown in Figures 2 to 4, which represent three different fields from the same microscopic preparation. Aggregates of viral antigen were characteristically revealed by fluorescence in the cytoplasm and around the nucleus of infected cells. Cytoplasmic fluorescence appeared mostly as pin-point granules, while perinuclear fluorescence was made up of heavy aggregates and sometimes diffuse masses. Occasionally, fluorescent granules were observed on top of the nucleus of infected cells probably because of the three-dimensional structure of the cell. Background nonspecific staining was minimal and is demonstrated in Figure 4 which was photographed with a long exposure time to permit the recording of the blue emission of host tissue autofluorescence along with the specific yellow-green fluorescence. Staining of uninfected (control) cells under similar conditions revealed no observable fluorescence (Figure 5) except for a diffuse tinge of blue tissue autofluorescence indicating the presence of HeLa cells.

Autofluorescence of host cells and tissue debris can be effectively quenched by counterstaining with 0.05% aqueous Evans blue. This dye fluoresces red under ultraviolet light and thus provides a good contrast to the specific yellow-green fluorescence (Fry and Wilkinson, 1963). By the use of Evans blue, the background can be counterstained orange-red and the observation of specific fluorescence is greatly facilitated.

Figure 2

Coxsackievirus B4-infected HeLa Cells Stained with Anti-coxsackievirus B4 Conjugate (Dilution 1:40)

Characteristic 4+ staining reaction showing aggregates of viral antigen in the cytoplasm and around the nucleus of infected cells. Cytoplasmic fluorescence appeared mostly as pin-point granules, while perinuclear fluorescence was made up of heavy aggregates and sometimes diffuse masses. Fluorescent granules occasionally were observed on top of the nucleus of infected cells probably because of the three-dimensional structure of the cell. Background nonspecific staining was minimal. x 250

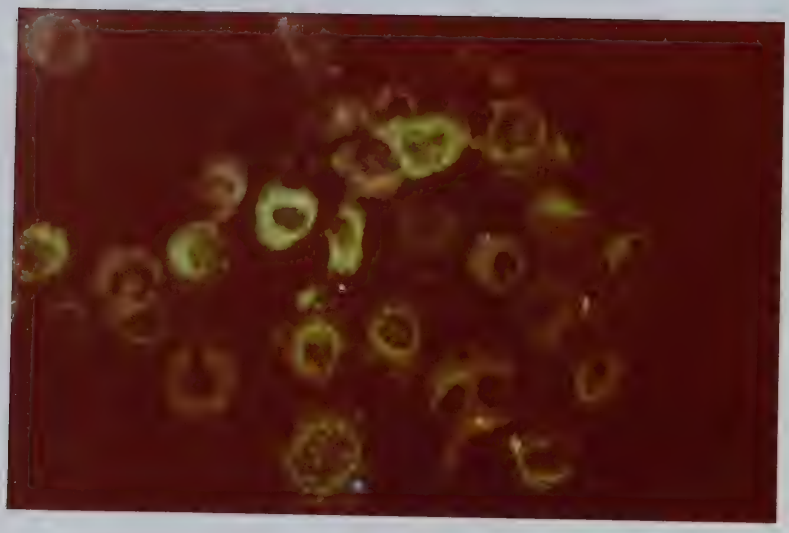
(Photograph courtesy of Dr. F.L. Jackson)

Figure 3

Coxsackievirus B4-infected HeLa Cells Stained with Anti-coxsackievirus B4 Conjugate (Dilution 1:40)

Another field from the same preparation as in Figure 2, showing 4+ staining reaction. x 250

(Photograph courtesy of Dr. F.L. Jackson)



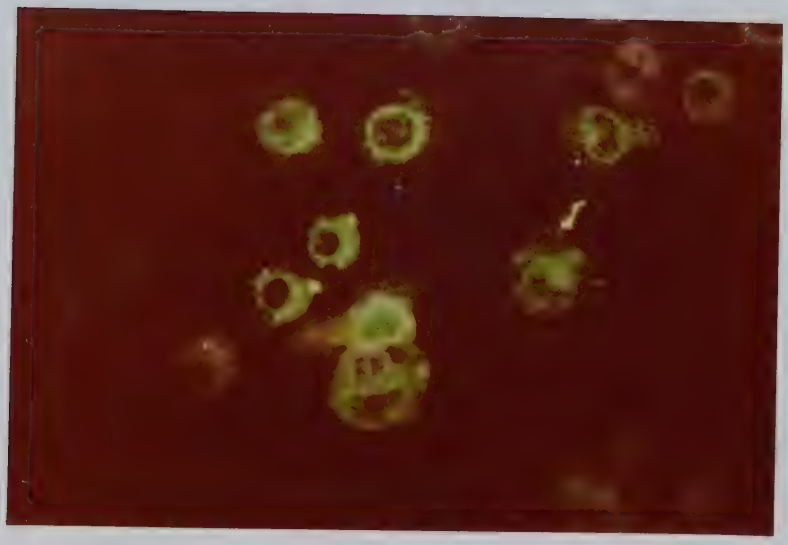


Figure 4

Coxsackievirus B4-infected HeLa Cells Stained with Anti-coxsackievirus B4 Conjugate (Dilution 1:40)

Another field from the same preparation as in Figure 2, photographed with long exposure to record the blue tissue autofluorescence of host cells as well as the specific yellow-green fluorescence of viral antigen. x 250

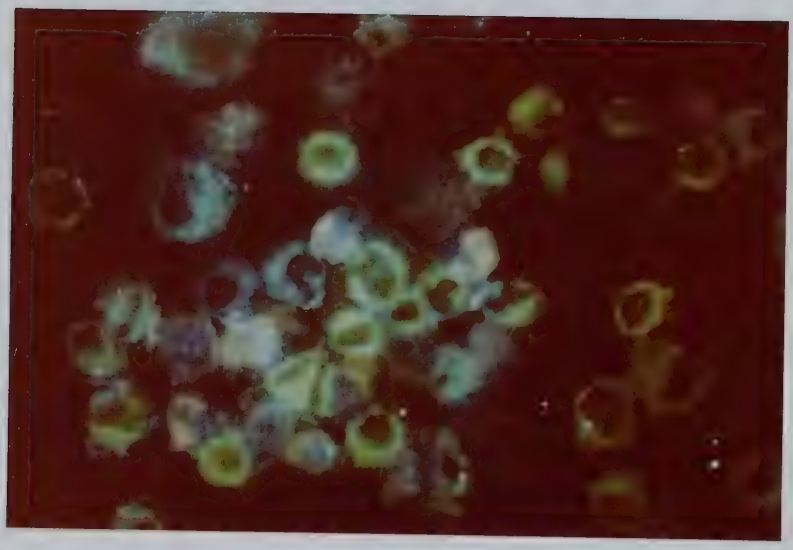
(Photograph courtesy of Dr. F.L. Jackson)

Figure 5

Uninfected (Control) HeLa Cells Stained with Anti-coxsackievirus B4 Conjugate (Dilution 1:40)

Negative staining reaction showing only traces of blue tissue autofluorescence indicating the presence of HeLa cells. x 250

(Photograph courtesy of Dr. F.L. Jackson)



Figures 6 to 9 illustrate the effect of Evans blue on infected and uninfected HeLa cells stained with the homologous conjugate. The red color in the background was due to Evans blue counterstaining.

In the staining of coxsackie B virus antigen with the homologous conjugate, two types of fluorescence were characteristically observed: intense fluorescence surrounding the nucleus and pin-point foci of fluorescence throughout the cytoplasm of infected cells. The intense perinuclear fluorescence was sometimes observed as aggregates of fluorescent granules and sometimes as a diffuse fluorescent mass at the immediate perinuclear area. The appearance of both perinuclear and cytoplasmic fluorescence has already been shown in Figures 2 to 4, but with Evans blue counterstaining, the yellow-green fluorescence became more outstanding. Figure 6 illustrates a 4+ homologous staining reaction in which coxsackievirus B4-infected HeLa cells were stained with anti-coxsackievirus B4 conjugate at dilution 1:40 and counterstained with Evans blue. Both the intense perinuclear fluorescent aggregates and pin-point cytoplasmic fluorescence were observed, although the latter was difficult to reproduce in photomicrography and the yellow-green fluorescence appeared more yellow than green when recorded on color films. The bright fluorescence stood out in marked contrast to the red uninfected cells and dark cell-free background. Uninfected (control) HeLa cells stained with anti-coxsackievirus B4 conjugate at dilution 1:40 are shown in Figure 7. The normal cells fluoresced in red because of Evans blue counterstaining.

A vivid illustration of the pin-point type of cytoplasmic fluorescence is given in Figure 8, which represents a 4+ homologous staining reaction involving coxsackievirus B1 antigen and anti-

Figure 6

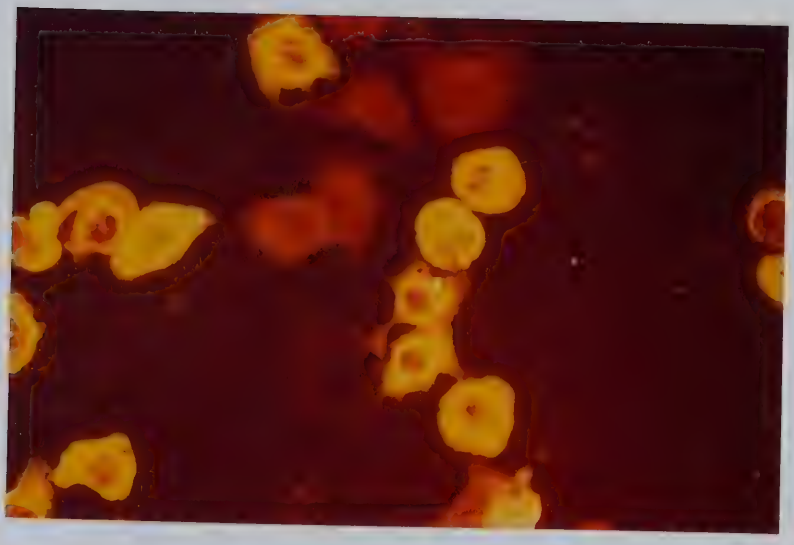
Coxsackievirus B4-infected HeLa Cells Stained with Anti-coxsackievirus B4 Conjugate (Dilution 1:40) with Evans Blue Counterstaining

Characteristic 4+ staining reaction showing intense perinuclear fluorescence and pin-point cytoplasmic fluorescence. The yellow-green fluorescence contrasted strongly against the uninfected HeLa cells (red) and cell-free background (dark). x 250

Figure 7

Uninfected (Control) HeLa Cells Stained with Anti-coxsackievirus B4 Conjugate (Dilution 1:40) with Evans Blue Counterstaining

Negative staining reaction showing uninfected HeLa cells counterstained orange-red by Evans blue. x 250



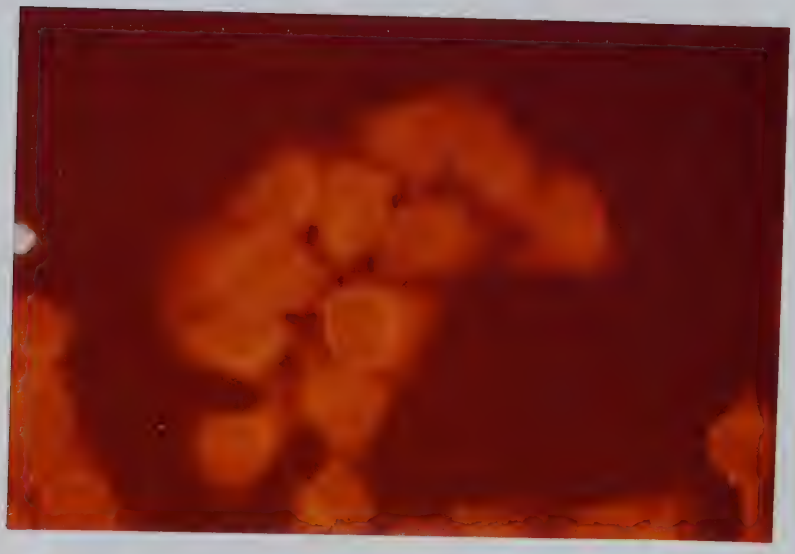


Figure 8

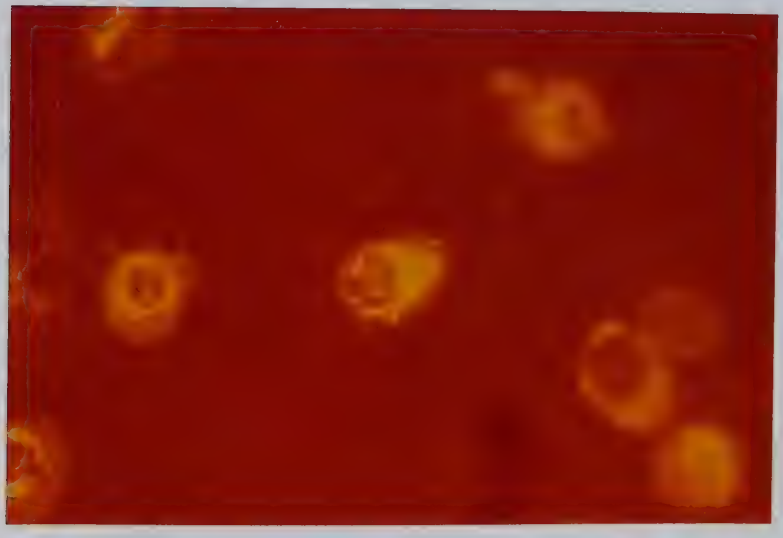
Coxsackievirus B1-infected HeLa Cells Stained with Anti-coxsackievirus B1 Conjugate (Dilution 1:40) with Evans Blue Counterstaining

Characteristic 4+ homologous staining reaction showing pin-point foci of cytoplasmic fluorescence together with a diffuse perinuclear fluorescent mass in the same infected HeLa cell (center). x 250

Figure 9

Coxsackievirus B1-infected HeLa Cells Stained with Anti-coxsackievirus B1 Conjugate (Dilution 1:40) with Evans Blue Counterstaining

4+ homologous staining reaction showing intense, diffuse perinuclear fluorescent masses in infected HeLa cells. x 250



coxsackievirus B1 conjugate at dilution 1:40. The fine, pin-point fluorescent granules characteristic of homologous staining were even more outstanding when viewed under the fluorescence microscope. Perinuclear fluorescence could also be observed in the same infected cell as a diffuse fluorescent mass surrounding the nucleus. The cell-free background was counterstained orange-red with Evans blue.

Figure 9 illustrates another example of a 4+ homologous staining reaction involving the same system (coxsackievirus B1 stained with anti-coxsackievirus B1 conjugate, dilution 1:40). Perinuclear fluorescence was more prominent than in Figure 8 and was observed as an intense, diffuse mass surrounding the nucleus. Cytoplasmic fluorescence, however, was less well demonstrated in this section of the coverslip, being represented by a few fluorescent granules scattered in the cytoplasm. The nuclei of infected HeLa cells appeared red and negative, marked occasionally with some fluorescent granules. The latter was regarded as cytoplasmic fluorescence observed on top of the nucleus because of the three-dimensional structure of the cell.

The presence of intense perinuclear fluorescence and pin-point cytoplasmic fluorescence in infected cells was characteristic of homologous staining for each of the coxsackie B viruses. Both types of fluorescence were therefore used as the criteria for evaluating specific staining.

Staining of coxsackie B virus antigen with conjugates heterologous to the antigen was characterized by a diffuse cytoplasmic fluorescence of 2+ or lower intensity observed only at high concentrations of the conjugate. This diffuse type of cytoplasmic fluorescence was readily distinguishable from the specific fluorescence described above.

Figure 10 shows a 1+ to 2+ diffuse cytoplasmic fluorescence demonstrated in the staining of coxsackievirus B1-infected HeLa cells with anti-coxsackievirus B2 conjugate at dilution 1:5. Perinuclear antigenic masses were vaguely discernible but were not clearly outlined and appeared to blend into the diffuse cytoplasmic fluorescence.

5. End-point Titration of Immunofluorescence Staining and Assessment of Cross-reactivity

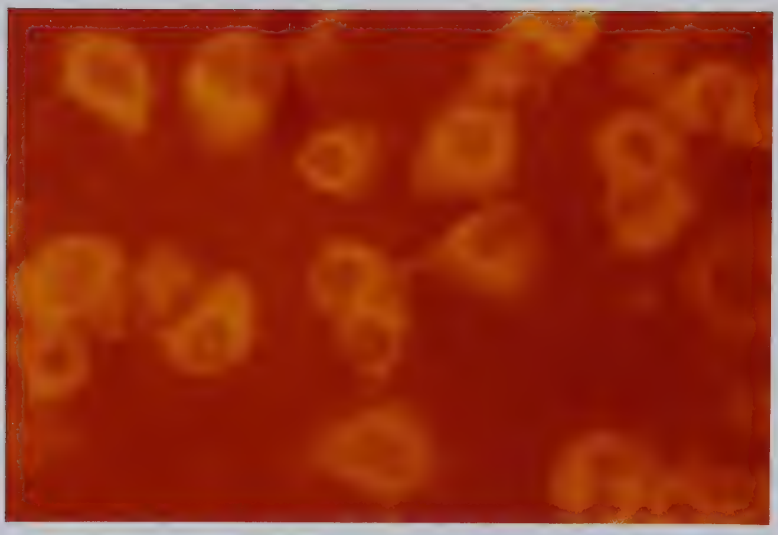
In order to evaluate the performance of immunofluorescent conjugates, a staining reaction of 3+ or 4+ was chosen as an end-point. The highest conjugate dilution which produced this end-point was regarded as the staining titer of the conjugate.

The staining sensitivity of each conjugate was assessed by determining its end-point titer using HeLa cells infected with the coxsackie B virus type homologous to the conjugate. Working dilutions 1:5 to 1:640 of the conjugate were prepared and used to stain the homologous antigen. On the other hand, the staining specificity of each conjugate was evaluated by testing the conjugate's ability to cross-react with heterologous coxsackie B viruses grown in HeLa cells. Working dilutions 1:5 to 1:80 of each conjugate were used to stain viral antigens heterologous to the conjugate. In every heterologous staining, a positive control of the viral antigen being stained was included. For instance, when coxsackievirus B4 was tested for cross-reaction with anti-coxsackievirus B1 conjugate, a positive control was included in which the presence of coxsackievirus B4 antigen was demonstrated by staining with the end-point dilution of anti-coxsackievirus B4 conjugate.

Figure 10

Coxsackievirus B1-infected HeLa Cells Stained with Anti-coxsackievirus B2 Conjugate (Dilution 1:5) with Evans Blue Counterstaining

Characteristic 1+ to 2+ diffuse cytoplasmic fluorescence of heterologous staining at high conjugate concentration. x 250



Tables 4 to 9 summarize the results of homologous and heterologous staining with conjugates to each of the coxsackie B viruses. The data represent composite results of many staining tests. Results of end-point titration and heterologous staining were repeatedly confirmed with different batches of coverslips and no more variation than half a grid on the 4 point scale was observed.

(a) Anti-coxsackievirus B1 Conjugate

Homologous and heterologous staining results of anti-coxsackievirus B1 conjugate are shown in Table 4. Staining of infected and uninfected HeLa cells at conjugate dilution 1:5 was frequently accompanied by a green "haze" in the background, which became rapidly diminished in the next dilution. Uninfected (control) cells stained at dilution 1:5 showed a light tinge of tissue autofluorescence of ± to 1+ intensity, but were counter-stained brown-red to red in subsequent dilutions. Occasionally, uninfected cells appeared brown-red and negative even when stained at dilution 1:5.

Two different kinds of fluorescence were observed in the staining of homologous (B1) viral antigen with anti-coxsackievirus B1 conjugate: perinuclear fluorescent masses and pin-point foci of fine cytoplasmic fluorescence. Both kinds of fluoresence were characteristic of specific staining. The conjugate had an end-point titer of 1:40 and was sensitive in staining its homologous viral antigen. The specific staining became better defined towards the end-point dilution, although the overall staining intensity was more glaring at lower dilutions. Staining of the homologous antigen at the end-point dilution was characterized

TABLE 4

HOMOLOGOUS AND HETEROLOGOUS STAINING

WITH ANTI-COXSACKIEVIRUS B1 CONJUGATE (F/P=6.5 µg/mg, molar F/P=2.7)

Conjugate Dilutions	Coxsackievirus B1		Coxsackievirus B2		Coxsackievirus B3		Coxsackievirus B4		Coxsackievirus B5		Coxsackievirus B6	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
1:5	4[a]	±	±	-	2[b]	1[b]	2[b]	1[b]	2[b]	1[b]	2[b]	±
1:10	4[a]	-	-	-	±	-	1[b]	-	1[b]	±	±	-
1:20	4[a]	-	-	-	-	-	-	-	-	-	-	-
1:40	4[a]	-	-	-	-	-	-	-	-	-	-	-
1:80	1-2[a]	-	-	-	-	-	-	-	-	-	-	-
1:160	±	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
1:320	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
1:640	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT

F/P: Fluorescein/Protein ratio
Test: Coxsackievirus-infected HeLa cells
Control: Uninfected HeLa cells
-: No fluorescence
±: Traces of fluorescence
1-2: Low level of fluorescence
3-4: Brilliant yellow-green fluorescence
a: Perinuclear and pin-point cytoplasmic fluorescence
b: Diffuse cytoplasmic fluorescence
NT: Not tested

by a 4+ specific fluorescence contrasted against a negative background where uninfected cells were counterstained orange-red and the intercellular matrix dark red. Staining of the homologous antigen at dilution 1:80 (next above the end-point titer) was markedly reduced, although characteristic perinuclear fluorescence and pin-point cytoplasmic fluorescence were still discernible. Consequently, end-point staining of the homologous antigen was readily distinguishable from staining at other dilutions. With increasing dilutions, the homologous staining became progressively diminished and was completely negative at dilution 1:640. At this point, infected and uninfected cells alike were counterstained orange-red.

Staining of heterologous antigens with anti-coxsackievirus B1 conjugate, dilution 1:5, revealed mostly 2+ diffuse cytoplasmic fluorescence which lacked the fineness of the specific pin-point fluorescence. Perinuclear fluorescence in heterologous staining was not clearly outlined and usually blended into the diffuse cytoplasmic fluorescence. Occasionally, as in the staining of coxsackievirus B2-infected cells, only a light tinge of diffuse cytoplasmic fluorescence was observed at dilution 1:5. The diffuse fluorescence rapidly disappeared at higher dilutions and was completely absent at dilution 1:20.

Contrary to the above observations, coxsackievirus B4-infected HeLa cells occasionally demonstrated a limited degree of cross-reactivity with anti-coxsackievirus B1 conjugate. Perinuclear fluorescence and pin-point cytoplasmic fluorescence of 2+ intensity were observed in a small number of coxsackievirus B4-

infected HeLa cells only when they were stained with the highest working concentration (dilution 1:5) of anti-coxsackievirus B1 conjugate. Although the quality of fluorescence resembled that of specific staining, the intensity (2+) was much lower than that of homologous staining at the same dilution. Furthermore, the majority of coxsackievirus B4-infected HeLa cells exhibited only a diffuse 2+ cytoplasmic fluorescence when heterologously stained with dilution 1:5 of anti-coxsackievirus B1 conjugate. The observation of perinuclear fluorescence and pin-point cytoplasmic fluorescence in the heterologous system was not entirely reproducible when different batches of coverslip preparations were tested. Consequently, it was not possible to decide whether the observation indicated a specific antigenic relationship between coxsackieviruses B1 and B4.

Based on the results of Table 4, it is evident that anti-coxsackievirus B1 conjugate was sensitive and specific in staining coxsackievirus B1 grown in HeLa cells. A limited degree of heterologous staining reactivities was observed only at high concentrations (dilutions 1:5 and 1:10) of the conjugate; at dilution 1:20, heterologous reactivities were completely eliminated. When used at its end-point dilution (1:40) to identify the homologous viral antigen, the conjugate was highly specific.

(b) Anti-coxsackievirus B2 Conjugate

Results of end-point titration and assessment of cross-reactivity for anti-coxsackievirus B2 conjugate are listed in Table 5. Staining of the homologous (B2) antigen showed a high

TABLE 5

HOMOLOGOUS AND HETEROLOGOUS STAINING

WITH ANTI-COXSACKIEVIRUS B2 CONJUGATE (F/P=6.3 µg/mg, molar F/P=2.6)

Conjugate Dilutions	Coxsackievirus B1		Coxsackievirus B2		Coxsackievirus B3		Coxsackievirus B4		Coxsackievirus B5		Coxsackievirus B6	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
1:5	2[b]	±	4[a]	±	2[b]	±	2[b]	±	1-2[b]	±	1[b]	±
1:10	1[b]	-	4[a]	-	1[b]	-	1[b]	-	±	-	±	-
1:20	-	-	4[a]	-	-	-	-	-	-	-	-	-
1:40	-	-	4[a]	-	-	-	-	-	-	-	-	-
1:80	-	-	4[a]	-	-	-	-	-	-	-	-	-
1:160	NT	NT	3[a]	-	NT	NT	NT	NT	NT	NT	NT	NT
1:320	NT	NT	2[a]	-	NT	NT	NT	NT	NT	NT	NT	NT
1:640	NT	NT	±	-	NT	NT	NT	NT	NT	NT	NT	NT

F/P: Fluorescein/Protein ratio
Test: Coxsackievirus-infected HeLa cells
Control: Uninfected HeLa cells
-: No fluorescence
±: Traces of fluorescence
1-2: Low level of fluorescence
3-4: Brilliant yellow-green fluorescence
a: Perinuclear and pin-point cytoplasmic fluorescence
b: Diffuse cytoplasmic fluorescence
NT: Not tested

end-point titer of 1:160 characterized by perinuclear and pinpoint cytoplasmic fluorescence. Heterologous staining of the other types of coxsackie B viruses revealed low levels of diffuse cytoplasmic fluorescence only at high conjugate concentrations (dilutions 1:5 and 1:10). Heterologous staining reactivities were rapidly eliminated by diluting the conjugate, while homologous staining remained brilliant up to the end-point dilution (1:160). When the conjugate was diluted to 1:20, it had no heterologous reactivity but still demonstrated 4+ homologous staining. Consequently, the conjugate could be effectively used at the end-point dilution (1:160) to identify its homologous viral antigen in HeLa cells.

(c) Anti-coxsackievirus B3 Conjugate

Evaluation of the homologous and heterologous staining properties of anti-coxsackievirus B3 conjugate can be seen in Table 6. The conjugate was very sensitive and demonstrated a high staining titer (1:80) for its homologous (B3) antigen in HeLa cells. When used at the highest working concentration (dilution 1:5) for heterologous staining, the conjugate showed only a 1+ to 2+ cytoplasmic fluorescence, exclusively of the diffuse type, with each of the heterologous types of coxsackie B viruses. At dilution 1:20, the conjugate possessed no heterologous staining reactivity. The conjugate at the end-point dilution (1:80) was therefore highly specific for the identification of its homologous antigen in culture cells.

TABLE 6

HOMOLOGOUS AND HETEROLOGOUS STAINING

WITH ANTI-COXSACKIEVIRUS B3 CONJUGATE (F/P=5.2 µg/mg, molar F/P=2.1)

Conjugate Dilutions	Coxsackievirus B1		Coxsackievirus B2		Coxsackievirus B3		Coxsackievirus B4		Coxsackievirus B5		Coxsackievirus B6	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
1:5	2[b]	±	1-2[b]	±	4[a]	1[b]	2[b]	1[b]	1[b]	±	2[b]	±
1:10	±	-	±	-	4[a]	-	1[b]	-	±	-	1[b]	-
1:20	-	-	-	-	4[a]	-	-	-	-	-	-	-
1:40	-	-	-	-	4[a]	-	-	-	-	-	-	-
1:80	-	-	-	-	3-4[a]	-	-	-	-	-	-	-
1:160	NT	NT	NT	NT	1-2[a]	-	NT	NT	NT	NT	NT	NT
1:320	NT	NT	NT	NT	±	-	NT	NT	NT	NT	NT	NT
1:640	NT	NT	NT	NT	-	-	NT	NT	NT	NT	NT	NT

F/P: Fluorescein/Protein ratio
Test: Coxsackievirus-infected HeLa cells
Control: Uninfected HeLa cells
-: No fluorescence
±: Traces of fluorescence
1-2: Low level of fluorescence
3-4: Brilliant yellow-green fluorescence
a: Perinuclear and pin-point cytoplasmic fluorescence
b: Diffuse cytoplasmic fluorescence
NT: Not tested

(d) Anti-coxsackievirus B4 Conjugate

Table 7 shows the results of homologous and heterologous staining with anti-coxsackievirus B4 conjugate. A high end-point titer (1:80) was again observed in the homologous staining system, characterized by both perinuclear and pin-point cytoplasmic fluorescence. On the contrary, staining of heterologous coxsackie B viruses at the highest working concentration (dilution 1:5) of the conjugate revealed only low levels of diffuse cytoplasmic fluorescence in infected HeLa cells. Heterologous staining reactivities were quickly eliminated by diluting the conjugate and were completely absent at dilution 1:20. As a result, the conjugate at the end-point dilution (1:80) was highly specific for detecting coxsackievirus B4 antigen in HeLa cells.

(e) Anti-coxsackievirus B5 Conjugate

In Table 8, the staining results of anti-coxsackievirus B5 conjugate are tabulated. The sensitivity of the conjugate was demonstrated by the high end-point titer (1:80) in homologous staining, which was characterized by perinuclear and pin-point cytoplasmic fluorescence. Heterologous staining reactivities consisted mainly of a 1+ to 2+ diffuse cytoplasmic fluorescence detected only at high conjugate concentrations (dilutions 1:5 and 1:10) and were effectively eliminated at dilution 1:20. The conjugate was thus highly specific at the end-point dilution (1:80) for identifying its homologous (B5) antigen.

TABLE 7

HOMOLOGOUS AND HETEROLOGOUS STAINING

WITH ANTI-COXSACKIEVIRUS B4 CONJUGATE (F/P=6.5 µg/mg, molar F/P=2.7)

Conjugate Dilutions	Coxsackievirus B1		Coxsackievirus B2		Coxsackievirus B3		Coxsackievirus B4		Coxsackievirus B5		Coxsackievirus B6	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
1:5	2[b]	1[b]	±	-	1[b]	±	4[a]	1[b]	1-2[b]	±	±	-
1:10	1[b]	-	-	-	-	-	4[a]	-	±	-	-	-
1:20	-	-	-	-	-	-	4[a]	-	-	-	-	-
1:40	-	-	-	-	-	-	4[a]	-	-	-	-	-
1:80	-	-	-	-	-	-	3-4[a]	-	-	-	-	-
1:160	NT	NT	NT	NT	NT	NT	1-2[a]	-	NT	NT	NT	NT
1:320	NT	NT	NT	NT	NT	NT	±	-	NT	NT	NT	NT
1:640	NT	NT	NT	NT	NT	NT	-	-	NT	NT	NT	NT

F/P: Fluorescein/Protein ratio
Test: Coxsackievirus-infected HeLa cells
Control: Uninfected HeLa cells
-: No fluorescence
±: Traces of fluorescence
1-2: Low level of fluorescence
3-4: Brilliant yellow-green fluorescence
a: Perinuclear and pin-point cytoplasmic fluorescence
b: Diffuse cytoplasmic fluorescence
NT: Not tested

TABLE 8

HOMOLOGOUS AND HETEROLOGOUS STAINING

WITH ANTI-COXSACKIEVIRUS B5 CONJUGATE (F/P=7.1 µg/mg, molar F/P=2.9)

Conjugate Dilutions	Coxsackievirus B1		Coxsackievirus B2		Coxsackievirus B3		Coxsackievirus B4		Coxsackievirus B5		Coxsackievirus B6	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
1:5	2[b]	±	1[b]	±	2[b]	1[b]	2[b]	1[b]	4[a]	±	±	-
1:10	1[b]	-	±	-	1[b]	-	1[b]	-	4[a]	-	-	-
1:20	-	-	-	-	-	-	-	-	4[a]	-	-	-
1:40	-	-	-	-	-	-	-	-	4[a]	-	-	-
1:80	-	-	-	-	-	-	-	-	3-4[a]	-	-	-
1:160	NT	NT	NT	NT	NT	NT	NT	NT	1-2[a]	-	NT	NT
1:320	NT	NT	NT	NT	NT	NT	NT	NT	±	-	NT	NT
1:640	NT	NT	NT	NT	NT	NT	NT	NT	-	-	NT	NT

F/P: Fluorescein/Protein ratio
Test: Coxsackievirus-infected HeLa cells
Control: Uninfected HeLa cells
-: No fluorescence
±: Traces of fluorescence
1-2: Low level of fluorescence
3-4: Brilliant yellow-green fluorescence
a: Perinuclear and pin-point cytoplasmic fluorescence
b: Diffuse cytoplasmic fluorescence
NT: Not tested

(f) Anti-coxsackievirus B6 Conjugate

Table 9 summarizes the staining results of anti-coxsackievirus B6 conjugate. The same general pattern of homologous and heterologous staining was observed as with other conjugates. Staining of the homologous antigen (B6) was characterized by perinuclear and pin-point cytoplasmic fluorescence as well as a high end-point titer (1:160). Staining of heterologous antigens was only observed at high conjugate concentrations (dilutions 1:5 and 1:10) and consisted of low levels of diffuse cytoplasmic fluorescence. Heterologous staining reactivities were totally absent at dilution 1:20 and higher dilutions. The conjugate could be effectively used at the end-point dilution (1:160) to identify coxsackievirus B6 in HeLa cells.

(g) Conjugate to Normal Serum

As a control to the specificity of the staining observed, a conjugate prepared from normal (non-immune) guinea pig serum was examined for staining reactivities with coxsackie B viruses. The results are compiled in Table 10. Infected and uninfected HeLa cells alike demonstrated only traces of diffuse background fluorescence when stained with the highest working concentration (dilution 1:5) of the conjugate. The cells were completely negative at subsequent dilutions up to and including 1:80. Positive controls of the viral antigens being stained (established by staining each antigen with its homologous conjugate at the end-point dilution) revealed characteristic 4+ fluorescence of both perinuclear and pin-point cytoplasmic types. The normal conjugate therefore possessed no nonspecific staining reactivity

TABLE 9

HOMOLOGOUS AND HETEROLOGOUS STAINING

WITH ANTI-COXSACKIEVIRUS B6 CONJUGATE (F/P=6.7 µg/mg, molar F/P=2.8)

Conjugate Dilutions	Coxsackievirus B1		Coxsackievirus B2		Coxsackievirus B3		Coxsackievirus B4		Coxsackievirus B5		Coxsackievirus B6	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
1:5	1-2[b]	±	1-2[b]	±	2[b]	±	1[b]	±	1[b]	±	4[a]	1[b]
1:10	±	-	±	-	1[b]	-	-	-	-	-	4[a]	-
1:20	-	-	-	-	-	-	-	-	-	-	4[a]	-
1:40	-	-	-	-	-	-	-	-	-	-	4[a]	-
1:80	-	-	-	-	-	-	-	-	-	-	4[a]	-
1:160	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	3[a]	-
1:320	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	1-2[a]	-
1:640	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	-

F/P: Fluorescein/Protein ratio
Test: Coxsackievirus-infected HeLa cells
Control: Uninfected HeLa cells
-: No fluorescence
±: Traces of fluorescence
1-2: Low level of fluorescence
3-4: Brilliant yellow-green fluorescence
a: Perinuclear and pin-point cytoplasmic fluorescence
b: Diffuse cytoplasmic fluorescence
NT: Not tested

TABLE 10

STAINING OF COXSACKIE B VIRUSES

WITH CONJUGATE TO NORMAL SERUM (F/P=5.0 µg/mg, molar F/P=2.1)

Conjugate Dilutions	Coxsackievirus B1		Coxsackievirus B2		Coxsackievirus B3		Coxsackievirus B4		Coxsackievirus B5		Coxsackievirus B6	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
1:5	±	-	±	±	±	±	±	±	±	±	±	±
1:10	-	-	-	-	-	-	-	-	-	-	-	-
1:20	-	-	-	-	-	-	-	-	-	-	-	-
1:40	-	-	-	-	-	-	-	-	-	-	-	-
1:80	-	-	-	-	-	-	-	-	-	-	-	-

F/P: Fluorescein/Protein ratio
Test: Coxsackievirus-infected HeLa cells
Control: Uninfected HeLa cells
-: No fluorescence
±: Traces of fluorescence

with coxsackie B viruses.

(h) Summary of Staining Titers

Results from Tables 4 to 10 are condensed into Table 11 which summarizes the staining titers of the conjugates. Only the reciprocal of each end-point dilution is shown. Homologous end-point titers were printed in bold type and heterologous titers in regular print. Occasionally, when staining at dilution 1:5 revealed only diffuse fluorescence of ± intensity, the titer was indicated as <5. The homologous titers of the conjugates were quite similar mainly because the protein content of each conjugate was adjusted to 10 mg/ml before working dilutions were made. Comparing the homologous and heterologous titers, it is evident that each conjugate was sensitive and specific for identifying its homologous antigen. It was sensitive because of its high end-point titer (1:40 to 1:160). It was specific because, at the end-point dilution, the conjugate had no heterologous staining reactivity but still showed 3+ or 4+ homologous staining. The conjugate was sensitive because of the specificity of the antibody.

No consistent patterns of heterologous staining were observed with conjugates to the various coxsackie B virus types. Most of the heterologous staining reactivities may have been due to group antigens of coxsackie B viruses.

TABLE 11

HOMOLOGOUS AND HETEROLOGOUS IMMUNOFLUORESCENCE STAINING TITERS

OF CONJUGATES TO COXSACKIE B VIRUSES

Conjugates to	Uninfected HeLa Cells	HeLa Cells Infected With					
		Coxsackievirus B1	Coxsackievirus B2	Coxsackievirus B3	Coxsackievirus B4	Coxsackievirus B5	Coxsackievirus B6
Cox B1	<5[c]	40[a]/80[b]	<5[c]	5[c]	10[c]	10[c]	5[c]
Cox B2	<5[c]	10[c]	160[a]/320[b]	10[c]	10[c]	5[c]	5[c]
Cox B3	5[c]	5[c]	5[c]	80[a]/160[b]	10[c]	5[c]	10[c]
Cox B4	5[c]	10[c]	<5[c]	5[c]	80[a]/160[b]	5[c]	<5[c]
Cox B5	<5[c]	10[c]	5[c]	10[c]	10[c]	80[a]/160[b]	<5[c]
Cox B6	5[c]	5[c]	5[c]	10[c]	5[c]	5[c]	160[a]/320[b]
Normal Serum	<5[c]	<5[c]	<5[c]	<5[c]	<5[c]	<5[c]	<5[c]

Cox: Coxsackievirus

a: Reciprocal of highest dilution showing 3+ or 4+ perinuclear and pin-point cytoplasmic fluorescence

b: Reciprocal of highest dilution showing 1+ or 2+ perinuclear and pin-point cytoplasmic fluorescence

c: Reciprocal of highest dilution showing 1+ or 2+ diffuse cytoplasmic fluorescence

6. Evaluation of Staining Specificity By Blocking Tests

The immunologic specificity of homologous staining for each anti-coxsackie B virus conjugate was evaluated by the antibody blocking test (sequence method) of Coons and Kaplan (1950). The test is based on the principle that, if the observed fluorescence is due to the specific binding of conjugated immunoglobulins to the homologous antigen, pre-treatment of the antigen with unconjugated immune serum will inhibit the specific fluorescence. On the other hand, blocking with normal (pre-immunization) serum should have no inhibitory effect.

The results of blocking tests performed with normal serum, homologous immune serum and heterologous immune sera to the antigen being stained are summarized in Tables 12 to 17.

For each anti-coxsackie B virus conjugate, blocking of homologous staining using the immune serum homologous to the antigen was always complete at the end-point titer of the conjugate, being marked by the total inhibition of fluorescence staining. In most instances, blocking was complete even at conjugate dilution 1:10. At the highest working concentration (dilution 1:5) of each conjugate, there was persistently a remarkable reduction in immunofluorescence staining due to blocking. At this dilution, the intense perinuclear fluorescence and pin-point foci of cytoplasmic fluorescence, both characteristic of the staining of the homologous antigen, were reduced to such an extent that only a diffuse tinge (± to 1+ intensity) of cytoplasmic fluorescence remained. The typical appearance of specific fluorescence was lost and instead only a faint diffuse fluorescence remained. Perinuclear fluorescence, which would have been brilliant if uninhibited by blocking, was observed as a diffuse brown mass surrounding the nucleus of infected cells. However,

TABLE 12

BLOCKING TESTS (SEQUENCE METHOD) OF HOMOLOGOUS STAINING
FOR ANTI-COXSACKIEVIRUS B1 CONJUGATE (F/P=6.5 µg/mg, molar F/P=2.7)

Conjugate Dilutions	Coxsackievirus B1-infected HeLa Cells Treated With													
	Normal Serum		B1 Immune Serum		B2 Immune Serum		B3 Immune Serum		B4 Immune Serum		B5 Immune Serum		B6 Immune Serum	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
1:5	4[a]	±	1[b]	±	4[a]	±	4[a]	±	4[a]	±	4[a]	±	4[a]	±
1:10	4[a]	-	±	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-
1:20	4[a]	-	-	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-
1:40	4[a]	-	-	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-
1:80	2[a]	-	-	-	1-2[a]	-	1[a]	-	1-2[a]	-	1-2[a]	-	1[a]	-

F/P: Fluorescein/Protein ratio
Test: Coxsackievirus-infected HeLa cells
Control: Uninfected HeLa cells
-: No fluorescence
±: Traces of fluorescence
1-2: Low level of fluorescence
3-4: Brilliant yellow-green fluorescence
a: Perinuclear and pin-point cytoplasmic fluorescence
b: Diffuse cytoplasmic fluorescence

TABLE 13

BLOCKING TESTS (SEQUENCE METHOD) OF HOMOLOGOUS STAINING
FOR ANTI-COXSACKIEVIRUS B2 CONJUGATE (F/P=6.3 µg/mg, molar F/P=2.6)

Conjugate Dilutions	Coxsackievirus B2-infected HeLa Cells Treated With													
	Normal Serum		B1 Immune Serum		B2 Immune Serum		B3 Immune Serum		B4 Immune Serum		B5 Immune Serum		B6 Immune Serum	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
1:5	4[a]	±	4[a]	±	1[b]	±	4[a]	±	4[a]	±	4[a]	±	4[a]	±
1:10	4[a]	-	4[a]	-	-	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-
1:20	4[a]	-	4[a]	-	-	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-
1:40	4[a]	-	4[a]	-	-	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-
1:80	4[a]	-	3-4[a]	-	-	-	3[a]	-	3-4[a]	-	3-4[a]	-	3-4[a]	-

F/P: Fluorescein/Protein ratio
Test: Coxsackievirus-infected HeLa cells
Control: Uninfected HeLa cells
-: No fluorescence
±: Traces of fluorescence
1-2: Low level of fluorescence
3-4: Brilliant yellow-green fluorescence
a: Perinuclear and pin-point cytoplasmic fluorescence
b: Diffuse cytoplasmic fluorescence

TABLE 14

BLOCKING TESTS (SEQUENCE METHOD) OF HOMOLOGOUS STAINING
FOR ANTI-COXSACKIEVIRUS B3 CONJUGATE (F/P=5.2 µg/mg, molar F/P=2.1)

Conjugate Dilutions	Coxsackievirus B3-infected HeLa Cells Treated With											
	Normal Serum		B1 Immune Serum		B2 Immune Serum		B3 Immune Serum		B4 Immune Serum		B5 Immune Serum		B6 Immune Serum	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
1:5	4[a]	1[b]	4[a]	±	4[a]	1[b]	1[b]	±	4[a]	±	4[a]	±	4[a]	±
1:10	4[a]	-	4[a]	-	4[a]	-	±	-	4[a]	-	4[a]	-	4[a]	-
1:20	4[a]	-	4[a]	-	4[a]	-	-	-	4[a]	-	4[a]	-	4[a]	-
1:40	4[a]	-	4[a]	-	4[a]	-	-	-	4[a]	-	4[a]	-	4[a]	-
1:80	4[a]	-	3-4[a]	-	3-4[a]	-	-	-	3-4[a]	-	3-4[a]	-	3-4[a]	-

F/P: Fluorescein/Protein ratio
Test: Coxsackievirus-infected HeLa cells
Control: Uninfected HeLa cells
-: No fluorescence
±: Traces of fluorescence
1-2: Low level of fluorescence
3-4: Brilliant yellow-green fluorescence
a: Perinuclear and pin-point cytoplasmic fluorescence
b: Diffuse cytoplasmic fluorescence

TABLE 15

BLOCKING TESTS (SEQUENCE METHOD) OF HOMOLOGOUS STAINING
FOR ANTI-COXSACKIEVIRUS B4 CONJUGATE (F/P=6.5 µg/mg, molar F/P=2.7)

Conjugate Dilutions	Coxsackievirus B4-infected HeLa Cells Treated With											
	Normal Serum		B1 Immune Serum		B2 Immune Serum		B3 Immune Serum		B4 Immune Serum		B5 Immune Serum		B6 Immune Serum	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
1:5	4[a]	1[b]	4[a]	±	4[a]	±	4[a]	±	±-1[b]	±	4[a]	±	4[a]	±
1:10	4[a]	-	4[a]	-	4[a]	-	4[a]	-	-	-	4[a]	-	4[a]	-
1:20	4[a]	-	4[a]	-	4[a]	-	4[a]	-	-	-	4[a]	-	4[a]	-
1:40	4[a]	-	4[a]	-	4[a]	-	4[a]	-	-	-	4[a]	-	4[a]	-
1:80	3-4[a]	-	3[a]	-	3[a]	-	3-4[a]	-	-	-	3-4[a]	-	3-4[a]	-

F/P: Fluorescein/Protein ratio
Test: Coxsackievirus-infected HeLa cells
Control: Uninfected HeLa cells
-: No fluorescence
±: Traces of fluorescence
1-2: Low level of fluorescence
3-4: Brilliant yellow-green fluorescence
a: Perinuclear and pin-point cytoplasmic fluorescence
b: Diffuse cytoplasmic fluorescence

TABLE 16

BLOCKING TESTS (SEQUENCE METHOD) OF HOMOLOGOUS STAINING
FOR ANTI-COXSACKIEVIRUS B5 CONJUGATE (F/P=7.1 µg/mg, molar F/P=2.9)

Conjugate Dilutions	Coxsackievirus B5-infected HeLa Cells Treated With													
	Normal Serum		B1 Immune Serum		B2 Immune Serum		B3 Immune Serum		B4 Immune Serum		B5 Immune Serum		B6 Immune Serum	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
1:5	4[a]	±	4[a]	±	4[a]	±	4[a]	±	4[a]	±	±-1[b]	-	4[a]	±
1:10	4[a]	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-	±	-	4[a]	-
1:20	4[a]	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-	-	-	4[a]	-
1:40	4[a]	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-	-	-	4[a]	-
1:80	3-4[a]	-	3-4[a]	-	3-4[a]	-	3[a]	-	3[a]	-	-	-	3[a]	-

F/P: Fluorescein/Protein ratio
Test: Coxsackievirus-infected HeLa cells
Control: Uninfected HeLa cells
-: No fluorescence
±: Traces of fluorescence
1-2: Low level of fluorescence
3-4: Brilliant yellow-green fluorescence
a: Perinuclear and pin-point cytoplasmic fluorescence
b: Diffuse cytoplasmic fluorescence

TABLE 17

BLOCKING TESTS (SEQUENCE METHOD) OF HOMOLOGOUS STAINING
FOR ANTI-COXSACKIEVIRUS B6 CONJUGATE (F/P=6.7 µg/mg, molar F/P=2.8)

Conjugate Dilutions	Coxsackievirus B6-infected HeLa Cells Treated With													
	Normal Serum		B1 Immune Serum		B2 Immune Serum		B3 Immune Serum		B4 Immune Serum		B5 Immune Serum		B6 Immune Serum	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
1:5	4[a]	±	4[a]	±	4[a]	±	4[a]	±	4[a]	±	4[a]	±	±	-
1:10	4[a]	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-	-	-
1:20	4[a]	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-	4[a]	-	-	-
1:40	4[a]	-	3-4[a]	-	4[a]	-	3-4[a]	-	4[a]	-	4[a]	-	-	-
1:80	4[a]	-	3[a]	-	3[a]	-	3[a]	-	3-4[a]	-	3-4[a]	-	-	-

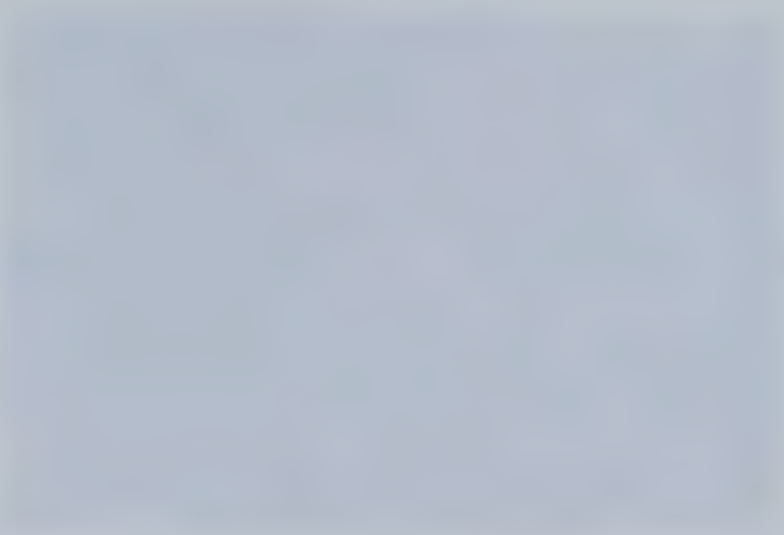
F/P: Fluorescein/Protein ratio
Test: Coxsackievirus-infected HeLa cells
Control: Uninfected HeLa cells
-: No fluorescence
±: Traces of fluorescence
1-2: Low level of fluorescence
3-4: Brilliant yellow-green fluorescence
a: Perinuclear and pin-point cytoplasmic fluorescence
b: Diffuse cytoplasmic fluorescence

the reduced perinuclear fluorescence appeared slightly yellow when the color slide was made into prints (Figure 11). On the other hand, the pin-point foci of cytoplasmic fluorescence were completely inhibited. When the blocked preparation was compared to the uninfected control cells treated and stained under similar conditions (Figure 12), it was almost completely negative.

On the contrary, blocking of homologous staining using immune sera heterologous to the antigen or using normal (pre-immunization) serum had no inhibitory effect on the brilliance of staining. Control blocking tests performed with normal (pre-immunization) serum had no effect on the end-point titer of each conjugate, indicating that the staining observed could not be inhibited by non-immunologic reactions. Failure of the heterologous immune sera to reduce the homologous staining reaction showed that a specific immune reaction was responsible for the staining observed.

The results of antibody blocking tests indicated that homologous staining by each conjugate was inhibited effectively only with the immune serum homologous to the antigen being stained and that blocking with normal serum or heterologous immune sera had no inhibitory effect. Consequently, the staining observed within each homologous system was considered immunologically specific.

Attempts have been made to block the heterologous staining reactions of each conjugate by the sequence method, using immune serum from which the conjugate was prepared and immune serum homologous to the antigen being stained. The usual normal serum control was included to ensure that no inhibition of staining was due to nonspecific factors. Heterologous staining reactions, characterized by a 1+ to 2+ diffuse



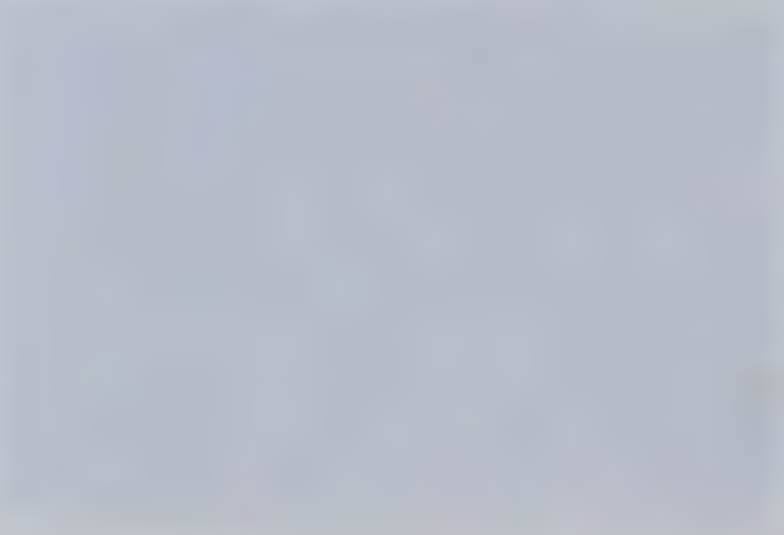


Figure 11

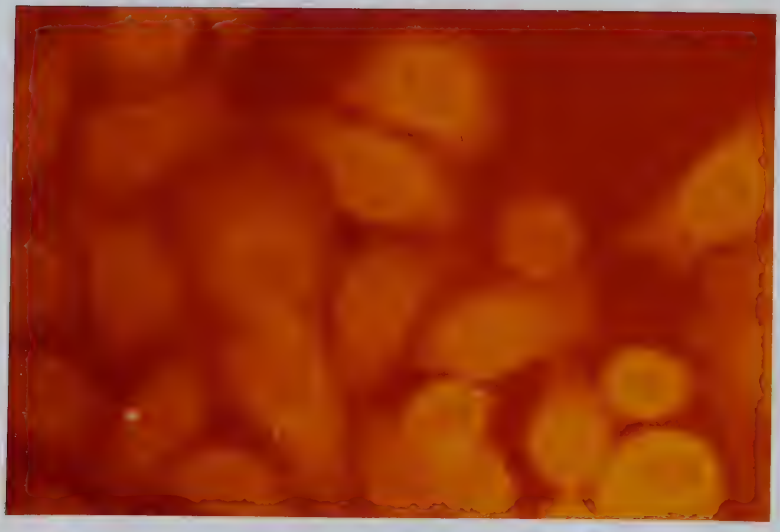
Blocking of Homologous Staining by Sequence Method (with Evans Blue Counterstaining)

Coxsackievirus B1-infected HeLa cells were treated with undiluted anti-coxsackievirus B1 immune serum and then stained with anti-coxsackievirus B1 conjugate at dilution 1:5. Perinuclear fluorescence was reduced to a diffuse brown mass and cytoplasmic fluorescence was completely inhibited. x 250

Figure 12

Uninfected HeLa Cell Control to Blocking of Homologous Staining (with Evans Blue Counterstaining)

Uninfected (control) HeLa cells were treated with anti-coxsackievirus B1 immune serum and then stained with anti-coxsackievirus B1 conjugate at dilution 1:5. Traces of diffuse tissue autofluorescence were observed. x 250



cytoplasmic fluorescence at high conjugate concentrations (dilutions 1:5 and 1:10), completely disappeared when immune serum from which the conjugate was prepared or immune serum homologous to the antigen was applied to the preparation before immunofluorescence staining. Blocking with normal (pre-immunization) serum did not reduce the diffuse fluorescence which remained vaguely discernible at high conjugate concentrations. This observation suggested that group antigens might be responsible for the heterologous staining reactivities observed among group B coxsackieviruses.

DISCUSSION

The observation that suckling mice were not susceptible to infection by the coxsackie B viruses prepared in this study suggested that the viruses had become adapted to propagation in tissue culture. This finding was not surprising because viruses have been shown to exhibit different biological properties when maintained in different hosts. Lehmann-Grube and Syverton (1961) reported that continuous passages in primary human amnion cell cultures of certain coxsackie A and coxsackie B viruses caused loss of virulence for newborn mice in varying degrees. However, most of the viruses reverted to their original virulence after 1 or 2 passages in the animal host. These findings led the above authors to suggest that a genetic mechanism was operative resulting in virus populations in which the majority of the particles was qualitatively changed. According to this hypothesis, the virus as it is propagated in suckling mice is pathogenic for mice. When the virus is passaged continuously in tissue culture, mutations occur giving rise to a variant with decreased mouse pathogenicity but increased virulence for cells in culture. This variant which is at a competitive disadvantage in mice rapidly outgrows the original type in culture cells.

One of the objectives of this study is to develop a reproducible method for the labelling of immunoglobulins with FITC. It is obvious from the nature of immunofluorescence that the degree of staining activity depends on the degree of conjugation. However, Frommhagen and Spendlove (1962) documented in studies of vaccinia virus-infected HeLa cells that overlabelling with FITC is the major cause of staining

nonspecificity. It is therefore important to label immunoglobulins to a suitable fluorescein/protein ratio in order to minimize nonspecific staining and yet retain bright specific staining.

The dialysis method of Clark and Shepard (1963) for FITC labelling has been shown to be definitely superior to the direct labelling method for preparing conjugates with minimal nonspecific staining (Hebert et al, 1967). The technique is based on the principle that, when FITC molecules are allowed to diffuse freely through a dialysis membrane into the globulin solution, uniform labelling at a suitable F/P ratio can be achieved provided that steady stirring is maintained throughout the process. Clark and Shepard's dialysis method prescribes that the globulin solution should be placed in a dialysis bag and dialyzed against 10 volumes of FITC solution. However, it is difficult to achieve uniform labelling by this method because the globulin molecules may not be sufficiently agitated inside the dialysis bag. On the suggestion of Dr. Morgante, a modification of the technique was brought about by reversing the dialysis arrangement, i.e., by placing the FITC solution in the dialysis bag and dialyzing it against a steadily agitated globulin solution. By carefully controlling the experimental conditions such as pH, temperature, reaction time and the concentrations of dye and protein used, a uniformly labelled conjugate may be obtained at a reproducible F/P ratio.

In this study, all immunoglobulin samples were labelled by the above modification of Clark and Shepard's dialysis method. Conjugation was conducted in the proportion of 18 mg of FITC per gram of protein on the basis of the findings of Spendlove (1966) as well as Clark and Shepard (1963). Both groups of workers have pointed out that a

concentration of FITC greater than 20 mg per gram of protein would lead to nonspecific staining. Dialysis was allowed to proceed at room temperature at pH 9.5, the optimum pH which gives the most rapid reaction without affecting the antibody properties (McKinney et al, 1964a). Conjugation was discontinued after 4 hours to avoid excessive labelling (Hebert et al, 1972). Conjugates prepared in the above manner varied only moderately in their F/P ratios, as the estimated molar ratios ranged from 2.1 to 2.9. The weight F/P ratios (µg/mg), ranging from 5.0 to 7.1, compare favorably in reproducibility with that of Clark and Shepard's preparations, which were in the range of 4.7 to 8.1.

There has been little agreement among workers in the field of immunofluorescence on the optimal F/P ratio of conjugates. Coons and Kaplan (1950) recommended labelling immunoglobulins at a molar F/P ratio of 1 to 2. The range of F/P ratio of the conjugates prepared in our study is in close agreement with Coons and Kaplan's initial recommendations. Since the performance of a conjugate relies on its staining sensitivity and specificity, the conjugates prepared in this study are deemed satisfactory in view of their high specific staining titer and low heterologous reactivity.

Various methods of measuring fluorescein/protein ratio have been described in literature. According to Beutner (1971), results of the field trial of six commercial conjugates by ten laboratories using a variety of methods for FITC and protein assays indicated that FITC assays performed by the method of McKinney et al (1964b) and protein assays by the biuret method (Gornall et al, 1949) yielded the most reproducible results. All F/P ratio determinations in this study were performed with the above mentioned procedures. In protein determinations, absorbance

was read at both 540 nm and 560 nm. However, only readings at 560 nm were used for the calculation of protein content in conjugates. This is because the presence of high concentrations of FITC in the protein-biuret reaction mixture will alter readings at the wavelength setting of 540 nm used for the original biuret method. Since the reaction mixture gives approximately the same readings at 540 nm and 560 nm, the slight shift in wavelength to 560 nm eliminates the interference of FITC absorption with the protein-biuret value (Hebert et al, 1972).

Weight F/P ratios in our study were converted to molar ratios by multiplying with the factor 0.411, as suggested by Hebert et al (1972). Since molar ratios are calculated from weight ratios and the calculation entails certain assumptions, the figures derived represent only estimated values. Consequently, both weight and molar F/P ratios were reported to define the conjugate.

The calculation of molar F/P ratios relies on the assumption that all of the protein is IgG, since the calculation is based on the molecular weight of IgG. By reporting the molar ratio, it is also assumed that the mean number of FITC molecules per IgG molecule equals the F/P ratio of the whole conjugate. In fact, IgG F/P ratios may not be equal to total F/P ratios, as most conjugates are not prepared from highly purified IgG. Nevertheless, even for conjugates prepared from crude ammonium sulfate-fractionated immunoglobulins, the molar F/P ratio still gives a reasonable estimate of the actual mean number of FITC molecules per IgG molecule (Beutner, 1971).

Nonspecific staining is probably the greatest technical problem encountered by workers in immunofluorescence. Fluorescence staining of microscopic preparations which is not due to specific reaction between

a particular antigen and its corresponding conjugated antibody is regarded nonspecific (Nairn, 1969). The occurrence of nonspecific staining interferes with the evaluation of specific staining and therefore must be differentiated from the latter and as far as possible eliminated. In this study, nonspecific staining was recognized as such by the staining of control (uninfected) HeLa cells in parallel with virus-infected cells. Additional controls for nonspecific staining were established by the use of a conjugate prepared from normal (non-immune) serum to stain HeLa cells with and without virus infection. It was by this double control of nonspecificity that specific fluorescence in excess of nonspecific fluorescence could be assessed.

Methods for the elimination or reduction of nonspecific fluorescence vary according to its source. Common sources of nonspecific fluorescence are : (1) unreacted fluorescent material (UFM) in the conjugate, (2) conjugated non-antibody serum proteins, and (3) unwanted conjugated antibodies (Nairn, 1969). Each factor will be considered individually and the corresponding methods used in this study to remove the nonspecific reactions will be discussed.

Unreacted fluorescent material present in the conjugate was removed by extensive dialysis, immediately following conjugation, against large volumes of 0.85% sodium chloride adjusted to approximately pH 8.5. Dialysis alone, however, was inadequate to free the conjugate of all unconjugated FITC. Complete removal of UFM was subsequently achieved by gel filtration with Sephadex G-50 Fine (Zwaan and van Dam, 1961). By this technique, rapid fractionation was achieved by differences in molecular size. The amount of protein lost on the column was negligible.

Nonspecific staining by conjugated non-antibody serum proteins is

mainly due to electrostatic forces between the tissue protein in the specimen and the conjugated molecules of serum proteins which are non-antibody in nature. At pH 7.0, the serum proteins have a net negative charge which is increased by conjugation. The conjugate then acts as an acid dye, staining the positively charged (basic) tissue proteins to produce nonspecific fluorescence. Absorption of conjugates with acetone-dried tissue powder has the effect of selectively removing molecules of high negative charge (Curtain, 1958). Absorption with tissue homogenates (Kaplan, 1958) would serve the same purpose. It is generally preferable to absorb with the tissue used for testing the conjugate. HeLa cells were used in the present study for the absorption of conjugates because all viral antigens were prepared in HeLa cells for immunofluorescence staining. Absorption of conjugates with HeLa cells has the additional advantage of simultaneously removing any unwanted conjugated antibodies, which constitute another common source of nonspecific staining.

The presence of non-antibody proteins in the serum has been shown to be an important source of nonspecific staining. Lewis et al (1964) and McKinney et al (1964a) reported that serum albumin binds FITC much more rapidly than globulin. The latter group of workers further indicated that the more rapid reaction of FITC with the albumin fraction would limit the amount of dye available to combine with the gamma-globulin. The efficiency of labelling of gamma-globulin would be greatly reduced and the F/P ratio of the gamma-globulin fraction would be difficult to control. They emphasized, therefore, the importance of removing such non-antibody serum proteins before conjugation. Moreover, when serum proteins are not evenly labelled with FITC, the molecules which are more heavily labelled acquire a higher negative charge and thereby

become a potent source of nonspecific staining. Consequently, nonspecific reactivity will be minimal when the conjugate consists only of specific antibody with a low uniform degree of molecular labelling (Nairn, 1969.) Conjugates prepared from highly purified IgG are thus preferable to that from whole serum. However, serum fractionation by a DEAE-cellulose column is a specialized procedure which can result in a considerable loss of specific antibody (Gardner and McQuillin, 1974). Highly purified IgG is therefore not frequently used for conjugation. In our study, immunoglobulins were prepared by ammonium sulfate precipitation, giving rise to a product of molecular heterogeneity containing mainly gamma-globulin and small amounts of alpha- and beta-globulins. Although these crude immunoglobulins were labelled without elaborate purification for IgG fractions, conjugates of considerably high staining specificity were obtained.

Another important source of nonspecific staining is tissue autofluorescence. Identification of virus antigen by immunofluorescence is performed in susceptible host cells, whether in vitro as in tissue culture cells or in vivo as in infected animal tissue sections. The natural fluorescence of most biological tissue is a mixture of the blue, blue-green and green fluorescent emissions of the various molecules making up the tissue. The phenomenon is referred to as autofluorescence. When animal tissue is exposed to ultraviolet or ultraviolet blue light and examined by darkground microscopy, the autofluorescence observed is largely blue (Nairn, 1969). The extent of tissue autofluorescence varies with the type of microscopic preparation. Tissue culture preparations, composed of one type of cells, emit less autofluorescence than animal tissue sections. Nevertheless, tissue autofluorescence is a major technical handicap to reckon with and is best quenched by background

counterstaining with other fluorescent dyes. The use of aqueous Evans blue for counterstaining FITC immunofluorescence was introduced by Nichols and McComb (1962) and recommended by Fry and Wilkinson (1963). However, Evans blue should be used at low concentrations because the dye has a detergent effect (Nichols and McComb, 1962). When used at high concentrations, Evans blue tends to remove insufficiently fixed tissue from the slide and may also obscure specific fluorescence. Trial runs in our study with and without Evans blue indicated that the use of Evans blue at a low concentration of 0.05% provided brilliant counterstaining, with no adverse effect on the specific staining titer of the conjugates.

In the present study, an aqueous solution of 0.05% Evans blue was applied for 5 minutes to coverslip preparations after immunofluorescence staining. Excess Evans blue was thoroughly removed by rinsing with PBS, pH 7.2, before mounting. When examined microscopically, HeLa cells counterstained with Evans blue emitted an orange-red fluorescence which provided a marked contrast with antigens specifically stained by FITC conjugates. All tissue autofluorescence was effectively masked. Evans blue was thus employed in all immunofluorescence staining in this investigation.

A principle factor of nonspecific staining is dye purity. The commercially available FITC dyes display a wide variation in purity. Hebert et al (1967) have shown that conjugates prepared with FITC of low purity exhibit greater nonspecific staining for a given FITC concentration than do those labelled with dyes of high purity. It is therefore desirable to use the purest FITC available in order to obtain conjugates with minimum nonspecific staining. Cherry et al (1969) extensively evaluated commercial FITC preparations and recommended that 70% FITC be accepted as

the minimal purity for immunofluorescence applications. In our study, FITC was purchased from Baltimore Biological Laboratory, Baltimore, Maryland. It contains not less than 80% pure dye (R. M. McKinney, personal communication).

The ultimate step in the elimination of nonspecific fluorescence is by dilution of the conjugate. When the specific staining titer is high, dilution of the fluorescein-protein conjugate provides an excellent means of reducing nonspecific protein interactions. As a standard practice in our experiments, the conjugate was first diluted to a protein concentration of 10 mg/ml, from which two-fold dilutions were prepared using a 10% mouse brain suspension. The highest conjugate dilution giving optimal specific staining was thereby determined.

Adjusting the conjugates to 10 mg/ml protein before dilution was necessary because conjugates varied slightly in their protein content. Without a standard protein concentration to start with, the end-point staining titers could not have been compared. The use of brain suspension (homogenized in PBS, pH 7.2) for conjugate dilution was suggested by French et al (1972) and strongly recommended in a recent report by Lennette and co-workers (1975). A 10% beef brain suspension was used by the latter group, who reported that nonspecific fluorescence disappeared when the brain suspension was used as diluent for the conjugate. In principle, the effect of the normal brain tissue is to selectively remove protein molecules of high negative charge. Normal mouse brain homogenate was used in our study because of its ready availability in our laboratory. In a series of preliminary tests, staining reactions with conjugates diluted in PBS (pH 7.2) and in a 10% mouse brain suspension were compared. The results indicated that, while specific staining titers of the conjugates

were virtually unaffected by the choice of diluent, staining of uninfected HeLa cells at dilution 1:5 without mouse-brain treatment revealed a higher degree of background nonspecific staining. Dilution of the conjugate in a 10% mouse brain suspension subsequently became part of our standard procedure.

The methodology used in the present study to prepare immunofluorescent reagents is based on the concept that immunoglobulins labelled by dialyzing the dye against protein for relatively short periods of time using dyes of high purity would yield conjugates of low nonspecific reactivity and high specific staining titer. From conjugation to its application to direct immunofluorescence staining, each conjugate went through a series of successive steps, namely: dialysis, HeLa cell absorption, Sephadex G-50 gel filtration, millipore membrane filtration, F/P ratio determination, storage at -70°C and dilution in a 10% mouse brain suspension immediately before use. Sephadex gel filtration was performed after HeLa cell absorption in order that cellular fragments could be retained by the Sephadex G-50 column. Subsequent filtration of the conjugate through a millipore membrane filter helped to achieve bacterial sterility, which is essential because contamination with microorganisms would affect the staining properties of the conjugate. After F/P ratio determination, the conjugate was stored at -70°C in small aliquots to prevent fading of the fluorochromes due to frequent freezing and thawing. No appreciable loss in the staining titer of conjugates in storage was experienced in this study. This may be partly attributed to the practice of preparing conjugates in small quantities (15 ml) in each conjugation procedure.

In search for a suitable cell system to prepare coverslip cultures

for infection by coxsackie B viruses, several tissue culture types have been tried, including both primary cell cultures and passaged cell lines. Initially, primary African green monkey kidney (AGMK) cells were chosen because of their high susceptibility and their being used in most diagnostic virology laboratories as the host system of choice for virus isolation. However, primary AGMK culture cells supplied by Connaught Laboratories and Flow Laboratories usually became degenerated in the long transit period required for the shipment of cells. The use of AGMK cells was subsequently discontinued.

On the contrary, passaged HeLa cells (human carcinoma of cervix) were excellent for preparing coverslip cultures by virtue of their high susceptibility, consistent cytological characteristics, ready availability in the laboratory and above all, the production of a monolayer practically free of cellular debris. Other cell lines had been experimented with, including the HEp-2 cells (human epidermoid carcinoma of larynx) and Vero cells (African green monkey kidney), but neither could match the HeLa cells in the morphological characteristics of grown cell layers. HeLa cells therefore became the cell of choice in preparing coverslip cultures.

In our study using coxsackie B virus-infected HeLa cells, two types of specific fluorescence were observed in homologous staining: intense perinuclear fluorescent masses and pin-point cytoplasmic fluorescence. Homologous staining titers of the conjugates ranged from 1:40 to 1:160, while heterologous staining revealed only low levels of diffuse cytoplasmic fluorescence at conjugate dilutions 1:5 and 1:10. Heterologous staining reactivity could therefore be eliminated by diluting the conjugate. At dilution 1:20, the conjugates possessed no detectable

heterologous staining reactivity but still displayed 4+ specific homologous staining. Each conjugate could thus be effectively used at its end-point dilution for the identification of the homologous viral antigen in HeLa cells. Background nonsepcific staining was minimal, indicating that the various methods used for its removal were highly successful. The direct immunofluorescent technique described in this study was therefore both sensitive and specific in identifying coxsackie B viruses in tissue culture cells.

The specificity of the fluorescence staining observed was established by the following criteria:

(1) Staining occurred only with intracytoplasmic viral antigen homologous to the conjugate.

(2) No staining was observed when anti-coxsakie B virus conjugates were applied to uninfected HeLa cells.

(3) No staining was produced by applying conjugated normal serum to coxsackie B virus-infected HeLa cells.

(4) Specific staining was completely inhibited by pre-treating the preparations with unconjugated immune serum homologous to the antigen being stained, but not by pre-treatment with heterologous immune sera or normal serum.

It was only by fulfilling these four conditions that any observed fluorescence could be considered specific.

No consistent patterns of cross-reactivity were noted among the coxsackie B viruses despite the presence of group antigens demonstrable by immunodiffusion and complement fixation tests (Schmidt et al, 1963 and

1965). The inability to detect consistent antigenic relationships among members of the coxsackie B virus group is probably due to variation in cultural characteristics of different batches of culture cells, which imposes a certain limitation on the reproducibility of fluorescence staining. While specific staining was recognized without failure, the detection of the intricate intertypic antigenic relationships among the group B coxsackieviruses was presumably beyond the sensitivity and specificity range of the immunofluorescent technique at its present state of development.

Perhaps the most significant finding of this investigation is the consistent observation of intense fluorescent masses around the nucleus of infected cells. These perinuclear fluorescent masses, believed to be aggregates of specific viral antigens, were observed consistently only in homologous staining. They remained clearly discernible even at a fluorescence intensity of 1+, when the infected cells were stained with high conjugate dilutions. Perinuclear fluorescence was absent in uninfected cells but was occasionally observed in heterologous staining at the highest working conjugate concentration (dilution 1:5). However, perinuclear fluorescence in heterologous staining lacked the clear-cut appearance of those in homologous staining and often blended into the diffuse cytoplasmic fluorescence. In blocking tests of specific staining, perinuclear fluorescence was quickly reduced to an orange-red mass when the viral antigen was treated with its homologous immune serum before staining. All these observations indicated that perinuclear fluorescent masses actually represent perinuclear aggregates of specific viral antigens. The occurrence of both perinuclear fluorescent masses and pin-point cytoplasmic fluorescence was therefore used as the guideline for

evaluating specific staining.

The sighting of perinuclear fluorescence in infected culture cells has rarely been reported in literature. Most immunofluorescence workers who studied enteroviruses grown in tissue culture have cited the observation of cytoplasmic fluorescent granules indicative of viral aggregates in the infected cells (Buckley, 1955; French et al, 1972). The description of perinuclear fluorescence in tissue culture system is unprecedented except for a brief mention in an early report. Mattern and Chi (1962) observed some faint perinuclear fluorescence in coxsackievirus A9-infected monkey kidney cells, but could not reproduce their findings. They dismissed the validity of their observations because similar fluorescence of lower intensity was occasionally observed in control cells. In studies of infected animal tissues, however, Rabin et al (1964) have demonstrated perinuclear fluorescence in an investigation of coxsackievirus B3 myocarditis in mice. These workers noted that, in sections of damaged myocardial fibers stained with FITC-conjugate, fluorescence was often intense in the immediate perinuclear area, although fluorescence was also detected throughout the cell. When examined by electron microscopy, numerous virus-like particles were found densely packed in the cytoplasm next to the nucleus where they sometimes appeared in the vicinity of nuclear pores. This distribution of particles corresponded to the perinuclear localization of viral antigen shown by immunofluorescence. In addition to the dense perinulcear location, particles were distributed in clusters throughout the cytoplasm of infected cells. These morphological observations led the above authors to suggest that the nucleus might play an active role in viral replication.

Validity of the perinulcear fluorescence observed in the present

study remains yet to be confirmed by other techniques. Immunofluorescence as a research tool has certain limitations. It needs to be supplemented by other techniques in order to elucidate the true nature and significance of these perinuclear fluorescent masses. Electron microscope studies have proved in the past to be useful in expanding the horizons of the immunofluorescent technique. Positive staining of thin sections of virus-infected HeLa cells will permit the visualization of virus particles at various sites in the infected cells. Examination of thin sections prepared at different hours after infection will provide information about the course of infection of culture cells by coxsackie B viruses.

Despite the recent advance of immunoperoxidase staining which challenges immunofluorescence staining as the rapid diagnostic method of choice, the immunofluorescent technique still has its place in clinical microbiology. It is valuable as a reference technique to standardize new diagnostic procedures by its high specificity and sensitivity. However, more quantitative standardization of the immune reagents is necessary because reproducibility of results can only be achieved through defined immunofluorescence staining.

SUMMARY AND CONCLUSIONS

A reproducible method for the conjugation of fluorescein isothiocyanate (FITC) to immunoglobulins was developed in this study for the rapid identification of coxsackie B viruses in tissue culture cells. The experimental procedures are briefly summarized as follows:

(1) Immune sera were obtained from guinea pigs immunized with coxsackie B viruses. Immunoglobulins were prepared by serum fractionation with saturated ammonium sulfate, followed by extensive dialysis against 0.85% sodium chloride to remove all traces of ammonium sulfate.

(2) FITC was conjugated to immunoglobulins in the proportion of 18 mg of FITC per gram of protein. Labelling was conducted by dialyzing the dye solution against the immunoglobulin solution at room temperature for 4 hours. The solutions were buffered at pH 9.5.

(3) Unreacted fluorescent material (UFM) in the conjugate was removed by dialysis against 0.85% sodium chloride. The conjugate was absorbed with HeLa cells and then filtered through a Sephadex G-50 column to remove various sources of non-specific staining.

(4) The protein content of the conjugate was determined by the biuret method. The amount of

protein-bound FITC was determined by using fluorescein diacetate as a reference standard. The fluorescein/protein ratio (F/P) of the conjugate was calculated and expressed in both weight (µg/mg) and estimated molar ratios.

The above procedures were used to prepare type-specific conjugates to each of the coxsackie B viruses as well as a normal (control) conjugate from non-immune serum. The conjugates had weight F/P ratios ranging from 5.0 to 7.1 and estimated molar ratios from 2.1 to 2.9. The relatively narrow range of F/P ratios indicated that the above procedures represented a reproducible method for FITC conjugation.

For application to direct immunofluorescence staining, each conjugate was adjusted to 1% protein (10 mg/ml), then diluted with a 10% mouse brain suspension and applied to air-dried, acetone-fixed HeLa cells (grown on coverslips) infected with coxsackie B viruses. The preparations were counterstained with 0.05% Evans blue to reduce tissue autofluorescence of host cells.

In the homologous staining of coxsackie B virus antigens, two types of specific fluorescence were observed: intense perinuclear fluorescent masses and pin-point foci of cytoplasmic fluorescence. Both types of fluorescence were used as the criteria for evaluating specific staining. Homologous staining titers of the conjugates varied from 1:40 to 1:160, while heterologous staining revealed only low levels of diffuse cytoplasmic fluorescence at conjugate dilution 1:5. At the end-point dilution, each conjugate possessed no heterologous staining reactivity but displayed brilliant (4+) specific staining.

The specificity of the fluorescence staining was established by

the following criteria:

(1) Staining occurred only with intracytoplasmic viral antigen homologous to the conjugate.

(2) No staining was observed when conjugates were applied to uninfected HeLa cells.

(3) No staining was produced by applying conjugated normal serum to virus-infected HeLa cells.

(4) Specific staining was completely inhibited by pre-treating the preparations with unconjugated immune serum homologous to the antigen being stained, but not by pre-treatment with heterologous immune sera or normal serum.

In conclusion, the immunofluorescence technique developed in this study was both sensitive and specific for the rapid identification of coxsackie B viruses in tissue culture cells. Adaptation of this technique for the detection of viral antigens in animal tissues appears highly promising.

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